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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**Patent Examining Operations**

Applicant: Johnson, S.

Serial No: 08/290,592

Art Unit: 1806

Filed: August 15, 1994

Examiner: Krikorian, J.

Title: Human-Murine Chimeric Antibodies Against Respiratory Syncytial Virus

Assistant Commissioner for Patents  
Washington, D.C. 20231

**Declaration of Leslie S. Johnson Under 37 CFR 1.131**

Dear Sir:

1. I, Leslie S. Johnson Ph.D., citizen of the United States of America, and residing at 13545 Ambassador Drive, Germantown, PA 20874; state and declare that upon information and belief that I am the inventor of the invention described and claimed in U.S. Serial No. 08/290,592, entitled "Human-Murine Chimeric Antibodies Against Respiratory Syncytial Virus".

2. This Declaration is submitted to establish conception of the invention in this application prior to March 1991, which is the effective date of Tempest *et al.*, cited by the Examiner and to establish reasonable diligence from a date prior to the effective date until Applicant's actual reduction to practice.

3. Attached hereto is Exhibit 1 which establishes a fully formed conception of the invention. It is an SBIR grant application (assignment number 1R43AI30300-01A1) which was submitted and receipt by the National Institutes of Health prior to March 1991. See particularly the bottom half of page 10 and the Experimental Design and Methods section at pages 14-18.

4. Attached hereto are Exhibits 2-4 which establish diligence in the completion of the invention from a time prior to the effective date of Tempest *et al.* continuously up to the date of the actual reduction to practice of the invention, by production of the humanized-mouse chimeric antibody. These documents are as follows:

Exhibit 2:

These notebook pages document work performed by Ms. Lisa Bennett on the cloning and manipulation of the murine anti-RSV MAb 1129 in preparation for humanization. Specifically detailed are the PCR amplification of the 1129 heavy and light variable regions, the insertion of these PCR fragments into plasmid vectors and the DNA sequencing of the resultant inserts. This work was particularly important because she confirmed the existence of additional variable region sequences from the parental myeloma cell line used to make the 1129 hybridoma. These pages document the construction of a single-chain Fv form of the molecule. The variable regions were then combined with the appropriate human constant region gene segment for eventual comparison to hu1129. Also the pages document the construction of a vector for the in-frame insertion of synthetic humanized V-region gene segments.

Exhibit 3:

These notebook pages documents work done by Mr. David Pfarr on the construction and evaluation of a eukaryotic expression vector capable of the expression of humanized anti-RSV MAb's. The vector needed to be capable of both transient and stable expression and to facilitate expression of heavy and light chain genes either by co-transfection or from the same plasmid; both under the control of a strong enhance/promoter.

**Exhibit 4:**

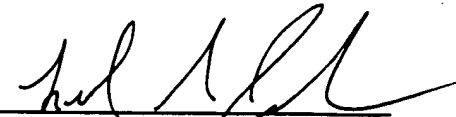
These pages document the expression and assay of humanized anti-RSV MAb hu1308F. Particularly they document the ability of hu1308F to bind to and thus immuno-precipitate radioactively labeled RSV F-protein expressed from a vaccinia virus construct. In addition they detail the transient expression of hu1129 and the generation of stable cell lines expressing hu1308F and hu1129.

5. This Declaration is submitted prior to final rejection.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereupon.

5/16/96

Date

  
Leslie S. Johnson, Ph.D.

**FIRST CLASS CERTIFICATE**

I hereby certify that this  
correspondence is being deposited today  
with the U.S. Postal Service as First  
Class Mail in an envelope addressed to:

Assistant Commissioner for Patents  
Washington, DC 20231

 5/21/96  
Charles J. Herron, Esq. Date

Respectfully submitted,



Charles J. Herron, Esq.  
Reg. No. 28,019

CARELLA, BYRNE BAIN, GILFILLAN,  
CECCHI, STEWART & OLSTEIN  
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Exhibit 1

FROM

IRG: SSS 4  
SPECIAL, SS 4  
DR. NABEEH MOURAD, EXEC. SECY.  
DRG/NATIONAL INSTITUTES OF HEALTH  
RM 2A04 5333 WESTBARD AVENUE  
BETHESDA, MD 20892 (301) 496-7310

A GRANT APPLICATION HAS BEEN RECEIVED BY NIH AND ASSIGNED TO AN INITIAL REVIEW GROUP (IRG) FOR SCIENTIFIC MERIT EVALUATION AND TO AN INSTITUTE FOR FUNDING CONSIDERATION. THE INITIAL PEER REVIEW SHOULD BE COMPLETED BY 12/90 AND A FUNDING DECISION SHOULD BE MADE SHORTLY AFTER THE APPROPRIATE NATIONAL ADVISORY GROUP MEETS IN 01/91. FOR QUESTIONS ABOUT THE ASSIGNMENT, CONTACT THE REFERRAL OFFICE (301) 496-7447. FOR QUESTIONS PRIOR TO THE REVIEW, CONTACT THE IRG EXECUTIVE SECRETARY OR THE INDIVIDUAL LISTED ABOVE. FOR QUESTIONS AFTER THE REVIEW, CONTACT THE INSTITUTE LISTED BELOW.

PRINCIPAL INVESTIGATOR: JOHNSON, LESLIE S COUNCIL: 01-91  
TITLE: HUMAN-MURINE CHIMERIC ANTIBODIES AGAINST RESPIRATORY SYN  
ASSIGNMENT NUMBER: 1R43A130300-01A1 IRG: SSS 4

NATL INST ALLERGY/INFECTIOUS DIS  
EXTRAMURAL ACTIVITIES RM 703  
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RESEARCH & DEVELOPMENT  
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DEPARTMENT OF HEALTH AND HUMAN SERVICES  
PUBLIC HEALTH SERVICE

DHB No. 0925-0195  
Revision Date 12/31/90

SMALL BUSINESS INNOVATION RESEARCH PROGRAM  
PHASE I GRANT APPLICATION

FOLLOW INSTRUCTIONS CAREFULLY

LEAVE BLANK  
ACTIVITY NUMBER  
REVIEW GROUP FORMERLY

COUNCIL/BOARD (Month/year) DATE RECEIVED

TITLE OF APPLICATION (Do not exceed 56 typewriter spaces)

Human-Murine Chimeric Antibodies Against Respiratory Syncytial Virus

1. SBIR SOLICITATION NO. PHS 90-2

3. PRINCIPAL INVESTIGATOR

2a. NAME (Last, first, middle)

Johnson, Leslie Sydnor

☐ New investigator

3b. SOCIAL SECURITY NO.

137-58-7149

2c. POSITION TITLE

Scientist

3d. MAILING ADDRESS (Street, city, state, zip code)

Molecular Vaccines, Inc.

19 Firstfield Road

Gaithersburg, MD 20878

2e. TELEPHONE (Area code, number and extension)

(301) 590-2622

4. HUMAN SUBJECTS

NO YES

☐ Exemption # \_\_\_\_\_  
OR  
☐ Form HHS 596 enclosed

5. VERTEBRATE ANIMALS

NO

YES

6. DATES OF PROJECT PERIOD

7. COSTS REQUESTED

a. Direct Costs

\$ 24,246

b. Total Costs

\$ 50,303

8. PERFORMANCE SITES (Organizations and addresses)

Molecular Vaccines, Inc.

19 Firstfield Road

Gaithersburg, MD 20878

9. APPLICANT ORGANIZATION (Name, address, and congressional district)

Molecular Vaccines, Inc.

19 Firstfield Road

Gaithersburg, MD 20878

6th Congressional District

10. ENTITY IDENTIFICATION NUMBER

52-1555759

11. SMALL BUSINESS CERTIFICATION

☒ Small business

☐ Minority and disadvantaged

☐ Woman-owned

12. NOTICE OF PROPRIETARY INFORMATION

The information identified by asterisks (\*) on pages \_\_\_\_\_

of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application; provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

13. DISCLOSURE PERMISSION STATEMENT

If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address and telephone number of the corporate official of your firm, to organizations that may be interested in contacting you for further information or possible investment?

YES NO

14. CORPORATE OFFICIAL

(Name, title, address and telephone number)

Dr. James F. Young

Vice President, Research & Development

Molecular Vaccines, Inc.

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Gaithersburg, MD 20878

Tel: (301) 590-2622

15. PRINCIPAL INVESTIGATOR ASSURANCE:

I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001).

SIGNATURE OF PERSON NAMED IN 3a  
(In ink. "P" or "r" signature not acceptable)

DATE

16. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willful false certification is a criminal offense (U.S. Code, Title 18, Section 1001).

SIGNATURE OF PERSON NAMED IN 14.  
(In ink. "Per" signature not acceptable)

DATE

## RESEARCH PLAN

### A. Specific Aims

Murine monoclonal antibodies against the F glycoprotein of RSV have been developed and characterized. These have been shown to neutralize RSV *in vitro* and protect cotton rats from lower respiratory tract infection *in vivo*. Previous studies with other rodent antibodies suggest that the development of human anti-mouse antibody responses would preclude the use of these murine antibodies directly in children. Therefore, it is the objective of this Phase I study to develop humanized versions of these anti-RSV antibodies which could then be used for passive immunization against RSV infection.

The specific objectives of Phase I work are as follows:

1. Clone and sequence the variable domains of the heavy and light chains from two selected murine anti-RSV monoclonal antibodies.
2. Substitute the coding regions for the complementarity determining regions from the murine cDNAs for the corresponding regions in human antibody heavy and light chain sequences.
3. Express the so constructed genes in mammalian cells and purify the humanized antibodies.
4. Characterize these antibodies with respect to:
  - a) antigen binding compared to the mouse parent
  - b) *in vitro* RSV neutralization, and
  - c) *in vivo* protection against RSV challenge in the cotton rat model

The successful completion of the studies described in this application will provide the basis for producing a product which could be tested in humans for prophylaxis against RSV infections in Phase II studies. It would also be prototypical for the development of humanized antibodies against other infectious agents and their use in treatment or prevention of illness.

### B. Significance

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infection in young children. In the United States alone, approximately 90,000 children are hospitalized and 4,500 children die from RSV infection each year (1). At special risk of serious RSV morbidity and mortality are children with underlying diseases such as congenital heart disease, bronchopulmonary dysplasia (BPD) and other pulmonary diseases, various congenital or acquired immunodeficiency syndromes and prematurity (2-5). There are over 100,000 such high risk children in the United States.

Infants with congenital heart disease (CHD) have a high fatality rate from RSV infection. In a prospective study, MacDonald *et al* (2) compared the course of hospitalized infants with CHD with and without RSV infection; the 27 CHD infants with RSV infection had a higher mortality rate (37%) both than the 46 CHD infants without RSV infection (6.5%) ( $p < 0.01$ ) and than the 202 infants without CHD but with RSV infection (1.5%) ( $p < 0.01$ ). In RSV infected infants with bronchopulmonary dysplasia, a chronic lung disease occurring in approximately 20% of all preterm infants requiring mechanical ventilation at birth (6), Groothuis *et al* (3) demonstrated a high degree of morbidity. Sixteen of 30 BPD patients followed prospectively over one winter were infected with RSV; 11 of them required hospitalization (4 in the intensive care unit). Although no fatalities occurred in this study, RSV is recognized as a common cause of death in BPD patients (7). Infants born

prematurely are a third and numerically important risk group for RSV infection, particularly during their first 6 months of life. In a nine year prospective study at the University of Rochester (5), 30% of children hospitalized with RSV pneumonia or bronchiolitis had been born prematurely. It is clear that RSV infection can be fatal in such infants (7).

Although an antiviral drug, ribavirin, has recently been licensed for therapy of RSV pneumonia and bronchiolitis (8,9), its value is controversial (10). Unfortunately, RSV vaccines are unavailable and unlikely to be licensed in the near future (1). A major obstacle to vaccine development is safety; the initial formalin inactivated RSV vaccine caused an increased incidence of RSV lower respiratory tract disease and death in immunized children (11). Currently the most promising approach to prophylaxis of RSV disease in high risk infants is passive immunization (12).

Support for the potential utility of passive immunization derives from studies of immune globulin or monoclonal antibodies in the prevention of experimental RSV infection in cotton rats (12-14) and Aotus monkeys (12, 15). In these animal models, immune globulin or monoclonal antibody infused prior to viral challenge prevented lower respiratory infection and reduced nasal RSV titers by 50-fold. Similar studies showed that high-titered RSV immunoglobulin was effective therapy for RSV infections in these animal models (13, 15). Infected animals treated with RSV immune globulin, showed no evidence of pulmonary immune-complex disease (13, 15). A double-blind, placebo-controlled intravenous immunoglobulin (IVIG) immunotherapy study was undertaken in 35 children hospitalized with RSV infection (16). Therapy was well tolerated and resulted in significant reductions in nasal RSV shedding and oxygenation in the treated infants. An NIAID sponsored multicenter Phase I safety trial of intravenous immune globulin given prophylactically to high risk infants was recently completed (17). Infusion of 10-15 ml/Kg of commercial lots of IVIG selected for high neutralizing antibody titer was generally well tolerated. RSV infection was detected in 9 of 21 patients, only one of whom had severe illness. Molecular Vaccines, Inc. ("MVI") is developing an RSV intravenous hyperimmune globulin for the prevention and treatment of RSV disease in high risk children. In collaboration with Massachusetts Public Health Biologic Laboratories, plasma from donors was screened by a virus micro-neutralization assay to identify those containing high titers of neutralizing antibody to RSV. These were then pooled and used to prepare a hyperimmune globulin which is currently being tested in a NIAID-sponsored multicenter Phase II efficacy trial for the prophylaxis of RSV lower respiratory tract infection in infants with serious heart and lung disease.

Even if RSV hyperimmune globulin is shown to reduce the incidence and severity of RSV lower respiratory tract infection in high risk children, several disadvantages may limit its use. One drawback is the necessity for intravenous infusion in these children who have limited venous access because of prior intensive therapy. A second disadvantage is the large volume of RSVIG required for protection, particularly since most these children have compromised cardiopulmonary function. A third disadvantage is that intravenous infusion necessitates monthly hospital visits during the RSV season which places these children at risk of nosocomial RSV infection (7). A final problem is that it may prove impossible to select sufficient donors to produce a hyperimmune globulin for RSV to meet the demand for this product. Currently only about 5% of normal donors have RSV neutralizing antibody titers high enough to qualify for the production of hyperimmune globulin.

One approach to circumventing a number of these problems may be to vaccinate adult volunteers with a live RSV vaccine to boost natural immunity in these individuals. This may then increase the number of individuals who have serum antibody titers high enough to be used to prepare a hyperimmune globulin, or to increase titers sufficiently high to prepare a product which could be used intramuscularly.

Another approach may be the development of monoclonal antibodies with high specific neutralizing activity as an alternative to hyperimmune globulin. The advantages of a monoclonal antibody approach are several, the most significant of which is that it would permit the production of a highly defined product with a standardized potency. Unlike an immunoglobulin product in which only a small fraction of the antibody is RSV-specific, a monoclonal antibody product would not contain any irrelevant antibody. Hence, the specific activity would be 50- to 100- fold higher than an immune globulin product and could potentially be used intramuscularly. In addition, if recombinant DNA methodology is used for production of the monoclonal antibody, it can be selectively engineered to provide the desired biological properties ( $F_c$  effector function, valency, pharmacokinetics, etc.) Monoclonal antibodies to other infectious agents for which these children are at risk (influenza and parainfluenza viruses) could also be combined to produce a "cocktail" and provide broader protection to these children. Since it is likely that repeated dosing of these monoclonal antibodies will be required during the high risk RSV season (November to March), it will be preferable, if not necessary, to use human monoclonal antibodies rather than murine or rat antibodies to minimize the development of human anti-rodent antibody responses which may compromise the therapeutic efficacy of the antibody or induce immune-complex pathology. However, the generation of human monoclonal antibodies with the desired specificity may be difficult and the level of production from human cell lines is often low, precluding their development.

An alternative is to produce human-mouse chimeric antibodies which contain the binding specificity of a pre-characterized murine antibody. A series of eighteen murine monoclonal antibodies against the RSV F glycoprotein have already been produced (18). They have been characterized with regard to their neutralizing potential, both to the parental RSV strains used to immunize the mice to generate the hybridomas, as well as a number of different virus strains isolated from 1956 to 1985 from various geographical locations. They have also been shown to provide protection in the cotton rat challenge model (Dr. Greg Prince, personal communication). In addition, these antibodies have been mapped by competitive binding and reactivity profiles of virus escape mutants to three broad antigenic sites (A, B and C) containing 16 epitopes. Epitopes within antigenic sites A and C show the least variability in natural isolates. MVI has obtained through Research Materials Transfer Agreements with Dr. Brian Murphy, National Institutes of Health and Dr. Larry Anderson, Centers for Disease Control, hybridomas producing eight of these murine monoclonal antibodies for further studies. These antibodies should were selected because they represented seven different epitopes, reacted with the highest number of virus strains tested (at least 13 out of 14 isolates) and retained the greatest neutralizing activity against virus escape mutants selected with the other antibodies. Table I summarizes the features of these eight antibodies.

Table I.  
Characteristics of murine monoclonal antibodies (31)

Monoclonal Antibody	Antigenic site	Epitope	Immuno-globulin subclass	# of RSV strains neutralized
1153	A	1	IgG1k	13/14
1142	A	1	IgG1k	13/14
1129	A	4	IgG1k	13/14
1121	A	5	IgG1k	13/14
1243	C	11	IgG2Ak	13/14
1332H	C	12	IgG2Ak	14/14
1308F	C	13	IgG1k	14/14
1302A	C	14	IgG1k	14/14

### Protection Against RSV Proliferation, *in vivo*

We examined eight of these monoclonal antibodies, specific for the RSV F glycoprotein, for their ability to block RSV replication when administered to cotton rats by the intranasal route prior to virus challenge. These antibodies were selected because they represented seven different epitopes, reacted with the highest number of virus strains tested (at least 13 of 14 isolates) and retained the greatest neutralizing activity against virus escape mutants selected with the other antibodies. Table I summarizes the features of these eight antibodies.

Cotton rats (*S. fulviventor*, 3 animals per group, average weight 100 grams) were anesthetized with methoxyflurane and given 0.1 ml of antibody solution (10 mg/ml or 1 mg/ml in phosphate-buffered saline (PBS) by intranasal (i.n.) instillation. A pooled monoclonal antibody mixture of all eight monoclonal antibodies (10 mg/ml total, 1.25 mg/ml each or 1 mg/ml total, 0.125 mg/ml each) was tested. Control animals were given 0.1 ml of a bovine serum albumin suspension (10 mg/ml or 1 mg/ml) in PBS or standard human immune globulin (Sandoglobulin, Sandoz, Inc., East Hanover, N.J.) in PBS (10 mg/ml or 1 mg/ml). One day later, animals were again anesthetized with methoxyflurane and challenged by i.n. instillation of  $10^5$  plaque forming units (PFU) of the Long strain of RSV. Four days after virus challenge, all animals were sacrificed by carbon dioxide asphyxiation. Lungs were harvested and homogenized in 10 parts (wt/vol) of Hanks balanced salt solution supplemented with 0.218 M sucrose, 4.4 mM glutamate, 3.8 mM  $\text{KH}_2\text{PO}_4$  and 3.2 mM  $\text{K}_2\text{HPO}_4$  and the resulting suspension was stored at  $-70^\circ\text{C}$  until assayed for virus content. Virus titers were determined using a plaque assay on HEP-2 cell monolayers and are expressed as PFU per gram of tissue.

The results shown in figure 1 indicate that each of the monoclonal antibodies showed a significant reduction in pulmonary virus titer, and were at least as effective and in most cases more effective than the standard immune globulin or the pool of the eight monoclonal antibodies. Surprisingly, five of the eight monoclonal antibodies appear to completely prevent infection at the high dose and one even at the low dose.

The eight MAbs described above were further examined for their ability to block RSV replication when administered at lower doses to cotton rats by the intranasal route prior to virus challenge. Cotton rats (*S. hispidus*, 4 animals per group, average weight 100 grams) were anesthetized with methoxyflurane and given 0.1 ml of antibody solution (1 mg/ml, 0.1 mg/ml or 0.01 mg/ml in phosphate-buffered saline (PBS) by intranasal (i.n.) instillation. Control animals were given 0.1 ml of a bovine serum albumin suspension (1 mg/ml) in PBS. One day later, animals were again anesthetized with methoxyflurane and challenged by i.n. instillation of  $10^{5.0}$  plaque forming units (PFU) of the Long strain of RSV. Four days after virus challenge, all animals were sacrificed by carbon dioxide asphyxiation. Lungs were harvested and homogenized in 10 parts (wt/vol) of Hanks balanced salt solution supplemented with 0.218 M sucrose, 4.4 mM glutamate, 3.8 mM  $\text{KH}_2\text{PO}_4$  and 3.2 mM  $\text{K}_2\text{HPO}_4$  and the resulting suspension was stored at  $-70^\circ\text{C}$  until assayed for virus content. Figure 4 shows that each of the monoclonal antibodies, except MAb 1243 showed a significant reduction in pulmonary virus titer when given at the 0.1 mg or 1 mg doses. MAb 1308 showed the best reduction among those to the C region of the F protein, while 1129 and 1121 were the best among those which bind to the A region.

Figure 1

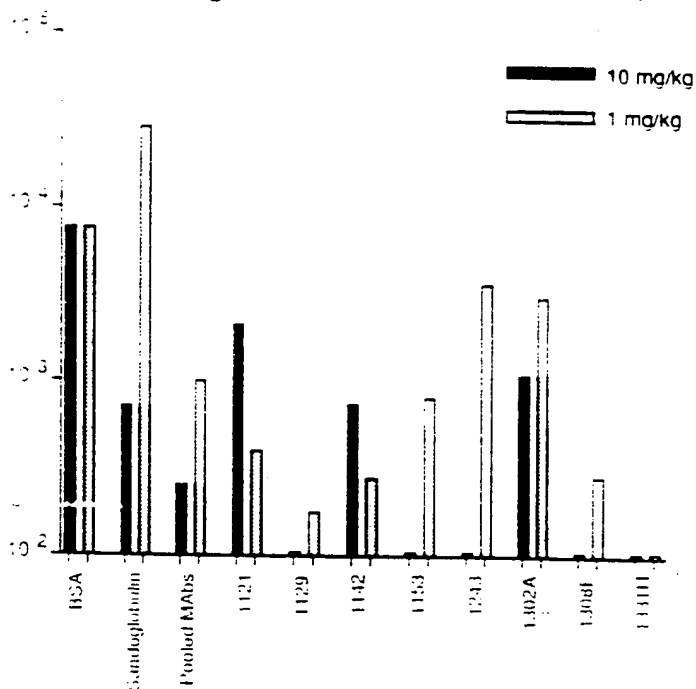


Figure 2

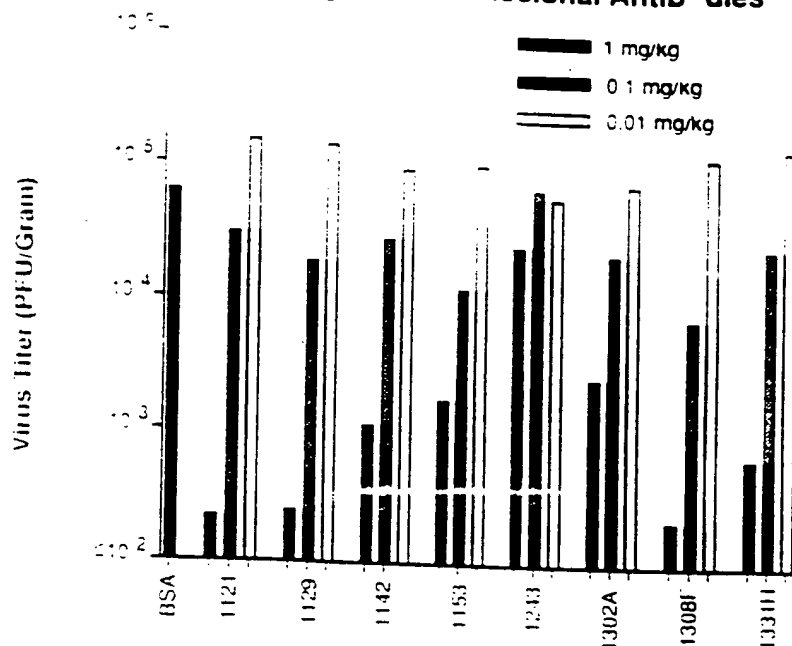


Figure 3

[illegible]

Figure 4

20 1119F Asp ~~Lys~~ ~~Lys~~ Met ~~Thr~~ ~~Thr~~ ~~Thr~~ Ser ~~Pro~~ ~~Ser~~ ~~Ser~~ ~~Met~~ ~~Thr~~ ~~Val~~ ~~Ser~~ ~~Leu~~ ~~Gly~~ ~~Glu~~ ~~Arg~~  
 CAC ATC AAG ATG ACC GAG TCT CCA TCT TCT ATG TAT TCT TCT CTA GGA GAG AGA  
 30 K102 Asp ~~Lys~~ ~~Val~~ Met ~~Thr~~ ~~Gln~~ Ser ~~Pro~~ ~~Ser~~ ~~Thr~~ ~~Leu~~ ~~Ser~~ ~~Ala~~ ~~Ser~~ ~~Val~~ ~~Gly~~ ~~Asp~~ ~~Arg~~  
 CAC ATC CAG ATG ACC GAG TCT TCT CCA TCT TCT GCA TCT CTA GGA GAG AGA  
 40 ~~Val~~ ~~Thr~~ ~~Thr~~ ~~Thr~~ ~~Cys~~ ~~Lys~~ ~~Ala~~ ~~Ser~~ ~~Gln~~ ~~Arg~~ ~~Thr~~ ~~Ala~~ ~~Thr~~ ~~Leu~~ ~~Ala~~ ~~Pro~~ ~~Phe~~ ~~Gln~~ ~~Gln~~  
 TCT ACT ATC ACT TGC AAG GCG ACT CAG CAC ATT AAT GAG TAT TTA AAC GCG TTC CAG CAG  
 50 ~~Val~~ ~~Thr~~ ~~Thr~~ ~~Thr~~ ~~Cys~~ ~~Arg~~ ~~Ala~~ ~~Ser~~ ~~Gln~~ ~~Ser~~ ~~Lys~~ ~~Ser~~ ~~Thr~~ ~~Leu~~ ~~Ala~~ ~~Pro~~ ~~Tyr~~ ~~Gln~~ ~~Gln~~  
 TCT ACT ATC ACT TGC AAG GCG ACT CAG ACT ATT AAT GAG TAT TTA AAC GCG TTC CAG CAG  
 60 ~~Lys~~ ~~Pro~~ ~~Gly~~ ~~Lys~~ ~~Ser~~ ~~Pro~~ ~~Lys~~ ~~Thr~~ ~~Leu~~ ~~Lys~~ ~~Ala~~ ~~Ser~~ ~~Ala~~ ~~Arg~~ ~~Val~~ ~~Ala~~ ~~Gly~~ ~~Val~~  
 AAA CCA GCG AAA TCT CTT AAG ACC CCG ATC CAT GAT GCA AAC ACA TTT GTA GAT GGG GTC  
 70 ~~Lys~~ ~~Pro~~ ~~Gly~~ ~~Lys~~ ~~Ala~~ ~~Pro~~ ~~Lys~~ ~~Leu~~ ~~Thr~~ ~~Asp~~ ~~Ala~~ ~~Ser~~ ~~Ser~~ ~~Leu~~ ~~Glu~~ ~~Ser~~ ~~Gly~~ ~~Val~~  
 AAA CCA GCG AAA CCG CTT AAG CTC CCG TTT ATG TAT GAT ACT GCG TCC GAA AGT GGG GTC  
 80 ~~Pro~~ ~~Ser~~ ~~Arg~~ ~~Phe~~ ~~Ser~~ ~~Gly~~ ~~Ser~~ ~~Gly~~ ~~Ser~~ ~~Gly~~ ~~Gln~~ ~~Glu~~ ~~Tyr~~ ~~Ser~~ ~~Leu~~ ~~Thr~~ ~~Ala~~ ~~Arg~~ ~~Ser~~ ~~Leu~~  
 CCA TCA AGG TTC ACT GGC AGT GGA TCT GGG GCA GAA GAA TAT TCT TCC ACC ATC ACC AGC Ser CCA  
 90 ~~Pro~~ ~~Ser~~ ~~Arg~~ ~~Phe~~ ~~Ser~~ ~~Gly~~ ~~Ser~~ ~~Gly~~ ~~Ser~~ ~~Gly~~ ~~Gln~~ ~~Thr~~ ~~Glu~~ ~~Phe~~ ~~Thr~~ ~~Leu~~ ~~Thr~~ ~~Lys~~ ~~Ser~~ ~~Ser~~ ~~Leu~~  
 CCA TCA AGG TTC AGC GGC AGT GCA TCT GGG ACA GAA TTT ACT TCT ACC ATC ACC AGC Ser CCA  
 100 ~~Thr~~ ~~Pro~~ ~~Glu~~ ~~Asp~~ ~~Met~~ ~~Gly~~ ~~Ile~~ ~~Tyr~~ ~~Tyr~~ ~~Cys~~ ~~Leu~~ ~~Glu~~ ~~Thr~~ ~~Gln~~ ~~Thr~~ ~~Pro~~ ~~Tyr~~ ~~Thr~~ ~~Phe~~  
 CAA TTT GAA GAT ATG GGA ATT TAT TAT TCT CTA CAG TTT CAT CAG TTT CCG TAC ACC TTT  
 110 ~~Gln~~ ~~Pro~~ ~~Asp~~ ~~Asp~~ ~~Phe~~ ~~Ala~~ ~~Thr~~ ~~Tyr~~ ~~Tyr~~ ~~Cys~~ ~~Gln~~ ~~Gln~~ ~~Tyr~~ ~~Asn~~ ~~Ser~~ ~~Tyr~~ ~~Ser~~  
 CAG CTT GAT GAT TTT GCA ACT TAT TAC TAC CAA GAT TAT AAT ACT TAT TCT  
 120  
 130 ~~Gly~~ ~~Gly~~ ~~Gly~~ ~~Thr~~ ~~Lys~~ ~~Leu~~ ~~Glu~~ ~~Ile~~ ~~Lys~~  
 GGA GGG GGC ACC AAG CTC GAA ATA AAA

### RSV Neutralization *in vitro*

The eight monoclonal antibodies were also tested for their ability to neutralize virus *in vitro*. Neutralization was measured by a plaque-reduction assay. The data are presented in Table 2. All antibodies showed neutralizing activity at the high concentration (10 mg/ml) whereas three of the monoclonal antibodies (1243, 1302A and 1331H) showed no activity at the lower concentration (1 mg/ml).

While there appears to be a correlation between *in vitro* neutralizing activity and *in vivo* protection for the antibodies to the C region of the F protein, such a correlation is not apparent for those to the A region.

Table II.  
Neutralization Titers for Monoclonal Antibodies

	<u>10 mg/ml</u>	<u>1 mg/ml</u>
BSA	<20	<20
Sandoglobulin	1360	200
Pooled MAbs	4100	300
1121	1340	120
1129	3540	240
1142	6140	420
1153	9940	700
1243	300	<20
1302A	480	<20
1308F	3540	440
1331H	1960	<20

Currently the murine antibodies are being further tested individually, and in pairwise combinations in the cotton rat for protective efficacy and potential synergy or competition. Two of these antibodies will then be selected, most probably one from antigenic site A and one from antigenic site C, to produce mouse-human chimeric antibodies. Although a number of laboratories have constructed chimeric antibodies with mouse variable and human constant domains (19-27), the mouse variable region may still be seen as foreign (28). Therefore, it is preferable to construct humanized antibodies in which only the hypervariable or complementarity determining regions (CDRs) of the heavy and light chains from the murine antibody of the desired specificity are transplanted onto the framework of a human antibody (28-30). These antibodies retain the binding capacity and affinity of the murine monoclonal antibody. Since the CDRs do not contain characteristic rodent or human motifs, these humanized antibodies should be indistinguishable in sequence from completely human antibodies thus minimizing human antibody responses. Once the humanized antibodies have been produced and characterized in these Phase I studies, Phase II studies would be initiated to examine the protective efficacy of these molecules in clinical studies.

### Cloning and Analysis of V-region cDNA's

On the basis of the above data, we selected the antibodies 1308F, specific for the C region of the RSV F protein, and 1129, specific for the A region as the initial targets for humanization. Total RNA from 1308F hybridoma cells was purified by the method of Chirgwin and subjected to one round of oligo dT chromatography. Double stranded cDNA was made from this RNA using primers complementary to the g1 or K constant region segment. The cDNA was inserted into pUC18 at the Sma I site and transformants were screened using a second oligonucleotide from a region of the c-region between the first strand cDNA primer and the variable region. cDNA inserts from positive clones were subjected to DNA sequencing and the variable region sequences were determined.



A comparison of the 1308F VH sequence with all sequences in the NBRF protein sequence database was performed using the wordsearch program from the University of Wisconsin analysis programs. This search revealed significant homology to two human VH regions. The highest homology was to the human germline gene HV3. The two sequences are 62% homologous overall and 65% in the framework regions. Significantly there is good homology at the junctions of the CDR segments and the frameworks with the exception of the 5' end of FR2. The mouse and human sequences as well as that of a potential CDR-Grafted combination of the two is shown in figure 3.

A similar analysis of the VL region revealed high homology to the human germ line V-kappa gene K102. The alignment of these sequences is shown in figure 2. In this case the homology is 62% overall and 73% in the framework regions. In each case a human J-region can be selected which is identical to the mouse sequence. This comparison is shown in figure 4.

### C. Relevant Experience

L. Syd Johnson (Ph.D.) is a molecular biologist with considerable experience in the cloning, sequencing, analysis and expression of antibody segments. While at Genex Corporation he was instrumental in the development of single-chain antibody technology.

Geetha Bansal (Ph.D.) has more than 10 years experience in the production of monoclonal antibodies, and in Western blotting, RIA, ELISA, receptor binding, T cell proliferation and lymphokine assays. She is currently involved in the development of parvovirus B19 diagnostic assays, vaccines and hyperimmune globulin as well as AIDS vaccine and AIDS immunotherapeutic research and development.

David Feller (Ph.D.) is a biological computational chemist with experience in writing and using computer applications for the purpose of structure-function analysis of proteins and nucleic acids.

Molecular Vaccines, Inc. is currently developing and testing hyperimmune globulin products for three human virus infections: respiratory syncytial virus (RSV), Parvovirus B19 and cytomegalovirus (CMV). Further, we have research, development and business relationships in place with Massachusetts Public Health Biologic Laboratories (Dr. George Siber) for production of these immunotherapeutic products. Clinical trials are in their second year for the RSV product. The CMV immunotherapeutic has been approved by the FDA and is expected to be marketed in

### D. Experimental Design and Methods

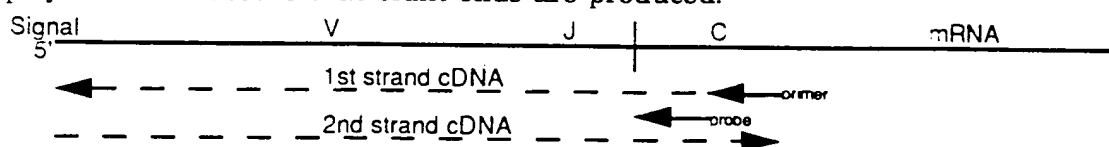
To develop humanized antibodies against RSV, we will begin with murine hybridoma cells producing murine monoclonal antibodies which have been shown to neutralize RSV *in vitro* and protect cotton rats against lower respiratory tract infection with RSV. RNA will be extracted from these cells, the rearranged immunoglobulin variable regions will be cloned and sequenced and murine CDR's will be substituted into human heavy and light chain cDNAs. The humanized antibodies will then be expressed and characterized relative to the parental murine antibodies to be certain that the genetic manipulation has not drastically altered the binding properties of the antibodies.

### 1. Cell Growth and mRNA Extraction

Murine Hybridoma cell lines will be maintained in RPMI 1640 supplemented with 15% fetal bovine serum. Cell culture supernatants will be routinely assayed by ELISA for the production of antibody with specificity for the RSV F protein. Total RNA from  $1 \times 10^8$  cells will be extracted by the method of Chirgwin, *et al* (32). pA<sup>+</sup> RNA will be purified by oligo-dT chromatography using spin columns. We have also had success purifying pA<sup>+</sup> RNA adequate for cDNA cloning of immunoglobulin variable regions using the Fast-Track kit from Invitrogen.

### 2. Variable Region cDNA Cloning and Sequencing

cDNA copies of the VH and VL of the target antibody will be generated essentially as described by Bedzyk, *et al* (33). The first strand cDNA reaction will be carried out using a phosphorylated oligonucleotide complementary to a segment of the mRNA coding for the constant region of the particular heavy or light chain isotype. This primer anneals to a segment of the mRNA adjacent to the variable region. Second strand cDNA synthesis will be carried out using RNase H and *E. coli* DNA polymerase I, as described by Gubler and Hoffman (34), followed by T4 DNA polymerase to assure that blunt ends are produced.



The ds-cDNA will then be ligated into pUC18 which has been digested with SmaI and treated with alkaline phosphatase. The ligated DNA will be used to transform *E. coli* DH5a by the method of Hanahan (35). Colony hybridization will be used to identify transformants carrying the desired cDNA segment. The probe for the hybridization will be a second segment of the c-region sequence lying between the first strand cDNA primer and the V-region. Probes corresponding to the J segments of the variable regions can also be used. Insert size of the plasmids in the positive clones will be determined. Because the 5' end of the first strand cDNA is fixed a nested set of sequences can be chosen for sequencing by picking those approximately 450bp (full length), 300bp and 150bp, subcloning into M13mp18 and mp19 and sequencing. After the sequence is determined in this way full length clones will be sequenced entirely on both strands to assure that this agrees with the composite sequence.

By taking advantage of the fact that immunoglobulin variable region genes are flanked by conserved sequences on one end and by semi-conserved sequences at the other, it is possible to design consensus primers to selectively clone the variable regions of rearranged immunoglobulin genes by the use of the polymerase chain reaction (PCR) (36,37). This approach will be used as an alternative if the cDNA cloning does not proceed rapidly, and as a replacement for this step at a later time.

### 3. Design and Assembly of Humanized Antibodies

The CDR regions of the V<sub>H</sub> and V<sub>L</sub> will be identified by comparing the amino acid sequence to known sequences as described by Kabat (38). In order to select the human framework sequences best able to accept the mouse derived CDR sequences in a conformation which retains the structure of the antigen combining site, the following strategy will be employed. First, the sequence of the murine V<sub>H</sub> region will be compared to known human sequences from both the Genbank and NBRF protein databanks using the Wordsearch program in the Wisconsin package of sequence manipulation programs (39). The best several human V-regions will then be

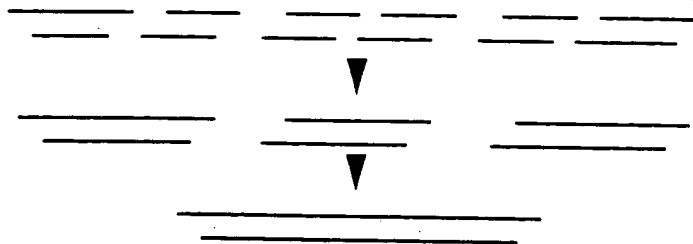
analyzed further on the basis of similarity in the framework regions, especially at the junctions of the framework and CDR regions.

Molecular modeling will be done using Silicon Graphics (SGI) computers at United States Army Research Institute of Infectious Diseases (USAMRIID) and at the Army's Ballistics Research Laboratory (BRL). The computer application that will be used is DISCOVER (Biosym Technologies, San Diego, CA) which is built upon the AMBER potential energy functions.

The human  $V_H$  regions selected in this way and the original mouse sequences will be modeled onto the corresponding region of another antibody for which a high resolution X-ray structure is available, such as MCP603. The murine CDR regions will then be inserted into the human model and the structures compared with particular attention to (a) the structure of the combining site and (b) possible perturbations of the framework structure. In cases where there is a divergence in the mouse and human sequences at the boundaries of a CDR both will be modeled. In general preference will be given to retaining the mouse residues in such a case if it appears likely that substitution of the human residues will perturb the CDR structure.

The human  $V_L$  region can be selected in one of two ways (a) Use the  $V_L$  which was associated with the human  $V_H$  used or (b) use the same strategy as was employed to select the human  $V_H$  framework. Which approach we use will depend on the availability of a both  $V_L$  and  $V_H$  sequences in the databases and relative homology of the two to the murine counterparts. The former approach is preferable if both sequences are available. In a case where a human  $V_H$  framework is found and the  $V_L$  is not available, such as where the human sequence is from an unrearranged germline gene, the latter strategy will be undertaken. As long as a  $V_L$  of the same subgroup as the one chosen has been found with a similar  $V_H$  such an approach should be valid.

The designed variable region genes will be assembled from oligonucleotides. Complementary pairs of 60mers with 10 base pair overlap will be made and annealed. Adjacent gene segments will be ligated together pairwise and then three sets will be combined to assemble an intact  $V_L$  or  $V_H$  gene segment (350bp).



#### 4. Expression of Humanized Antibodies

Once the humanized  $V_L$  and  $V_H$  have been designed and modeled the project becomes more empirical in nature. Genes will be designed synthesized and expressed, at which time the question of whether the affinity of the recombinant antibody is similar to the murine Mab from which it was derived can be answered. The rapidity with which these steps can be undertaken is thus of importance in finally obtaining a humanized antibody which retains most of the affinity of the parent. To this end we will work with transient expression systems to produce large enough amounts of antibody or antibody fragments to compare to the native antibody or fragments therefrom.

The ability of recombinant vaccinia virus carrying the bacteriophage T7 RNA polymerase gene to promote efficient expression of foreign genes co-introduced under the control of a T7 promoter will be utilized as our initial production system. Plasmid vectors containing a T7 promoter and a Cap-independent translation initiation signal from the encephalomyocarditis (EMC) virus are available from Dr Thomas Fuerst at MVI. We have recently used such a vector to produce an active single-chain antibody (SCA) at a level of approximately 2ug/ml. The active SCA appeared to have been secreted as evidenced by removal of the signal peptide. While we could also produce an SCA version of the chimeric antibody, we do not foresee the necessity of this step for this project, where the end product will be a full sized antibody. Instead we will co-express heavy and light chain genes either on the same plasmid or introduced on different plasmids. Production of whole immunoglobulins from a single recombinant virus has been achieved in insect cells using a baculovirus system (43). The utility of a rapid transient system depends on how much "tinkering" is required on the recombinant protein(s). If the initial design produces an antibody with acceptable binding and neutralization characteristics it could go directly into the final expression system. If, on the other hand, more modifications need to be made the utility of the transient expression system will be significant.

Stably co-transformed mouse myeloma cell lines or "transfectomas" will then be generated in order to provide an ultimate production source for the modified antibodies. This will be done in a manner similar to that described by Oi, *et al.* (44). The heavy and light chain hybrid human-mouse cDNAs will be separately inserted into different expression vectors containing Ig promoter/enhancer sequences, to drive transcription of the cDNAs (22,29-31), and the dominant selectable markers for G418 (neo) or mycophenolic acid (gpt).(22). The expression vectors for the heavy and light chain cDNAs will then be co-transfected (electroporated) into Sp2/0, a non-producing mouse myeloma cell line. Transfectants will be selected initially for G418 resistance; stable transfectants will then be selected in medium containing both G418 and mycophenolic acid to derive cell lines which have incorporated vectors for both the heavy and light chains.

Cell lines will then be screened for production of heavy and light chain proteins by radioimmunoprecipitation. Cell lines expressing both chains will then be expanded, supernatants will be collected and antibody will be purified by affinity chromatography on protein G (Gammabind plus, Genex, Inc.).

## 5. Characterization of Humanized Antibodies.

a. Antibody binding to the RSV F protein. The purified antibodies will be assayed by an ELISA for reactivity with the RSV F glycoprotein expressed by a recombinant vaccinia virus (18) or purified RSV virions. To evaluate the relative binding affinity of the chimeric antibodies as compared to the parental murine antibody, competition solid-phase radioimmunoassays will be performed (18). One antibody will be metabolically labeled with <sup>35</sup>S-methionine. A saturating amount of labeled antibody will be mixed with increasing concentrations of either the murine or chimeric antibodies and competition curves generated. Reciprocal competition experiments will also be performed. In this way inhibition curves can be compared and the relative binding affinity of the antibodies can be determined. This assay is in place at MVI and has been used to map MAB's to different antigenic sites.

b. In vitro virus neutralization. Virus neutralization curves will be determined in a standard assay. Serial two-fold dilutions of antibodies will be mixed with 100 tissue culture infectious dose<sub>50</sub> of RSV in the presence of 5% guinea pig complement. After

sixty minutes, the virus antibody mixtures will be transferred to Hep-2 cells in 96-well plates and six days later monolayers will be examined microscopically for cytopathology. Neutralization titers will be expressed as the lowest antibody concentration which inhibits more than 95% of viral cytopathic effect present in control wells.

c. In vitro protection studies in cotton rats. Cotton rats will be administered different doses of antibody 24 hours prior to intranasal challenge with RSV. Four days after challenge the animals will be sacrificed and viral titers in lung and nasal tissues will be determined by a standard plaque assay on Hep-2 cell monolayers as described (13). These studies will be done through a contractual arrangement with Jackson Foundation (see section H).

#### E. Human Subjects

None

#### F. Vertebrate Animals

The proposed project involves the use of adult cotton rats (*Sigmodon hispidus*) to assess the protective efficacy of antibodies against RSV challenge. Animals will be obtained from the Veterinary Resources Branch, Division of Research Services, National Institutes of Health. Approximately 45 animals will be used for these studies.

Animals will be housed at the Uniformed Services University of the Health Sciences or off-site at a contract facility on Medical Center Drive, in Gaithersburg, Maryland. An AALAC certified veterinarian is available to counsel of the care and use of animals. Veterinary care includes a program for prevention of disease; daily observation and surveillance for assessment of animals health; appropriate methods of disease control, diagnosis, and treatment; and guidance of animal users in appropriate methods of handling, restraint, anesthesia, and euthanasia.

All procedures in this project ensure minimal discomfort to animals. Animals will be anesthetized with methoxyflurane prior to intranasal instillation of antibody and virus challenge. At four days post-infection; animals will be sacrificed by carbon dioxide inhalation.

#### G. Consultants/Collaborators

(see section H).

#### H. Contractual Arrangements

MVI has established a collaborative research and development agreement with the Henry M. Jackson Foundation to develop and produce immunotherapeutic products to prevent or to treat serious respiratory infections caused by respiratory syncytial virus, parainfluenza viruses, and influenza. Participating from the Jackson Foundation are Drs. Val Hemming, Gerald Fisher, and Greg Prince who have pioneered the use of immunoglobulins in the therapy of respiratory syncytial virus infection. While the initial goal of the agreement is to develop high titered, enriched RSV immunoglobulin for intravenous use, second generation products are under development that are an improvement over intravenous immunoglobulin in regard to potency, duration/protection,

and ease of delivery. In addition, MVI has licensed from the U.S. Government exclusive rights for the topical (aerosolized) use of enhanced immune globulin for the therapeutic and prophylactic treatment of RSV infection. No support for this arrangement are sought though this SBIR proposal.

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**Other Grant and contract support**

**A. Active Research Support:**

1. NIAID, NCVDP; Contract No. AIDS-1,1U01 AI28171-01  
 "Vaccines Development for Human Immunodeficiency Viruses"  
 P.I.: Wayne T. Hockmeyer  
 % effort, G. Bansal: 40%  
                   D. Feller: 25%  
 Annual direct costs: \$592,261  
 Grant period: 3/89-2/94
2. DOD, USAMRDC; log No. 89166005  
 "Flarivirus RNA Replication: Essential Viral Functions as Targets for Antiviral Therapeutics"  
 P.I.: Marc S. Collett  
 %effort, G. Bansal: 10%  
                   D. Feller: 30%  
 Annual direct costs: \$216,633

**B. Proposals Pending Funding:**

1. NIH, Phase I SBIR  
 "Surrogate assay for B19 Parvovirus Serum Neutralization"  
 P.I.: Geetha Bansal: 30% effort  
 % effort, J. Hatfield: 20%  
 Annual direct costs: \$24,172

**D. Application Organization Active/Pending Funding for Work Related to this Project:**

None

Figure 1  
Pr phylaxis of RSV Infection in *S. Fulviventer*  
Using Anti-F Monoclonal Antibodies

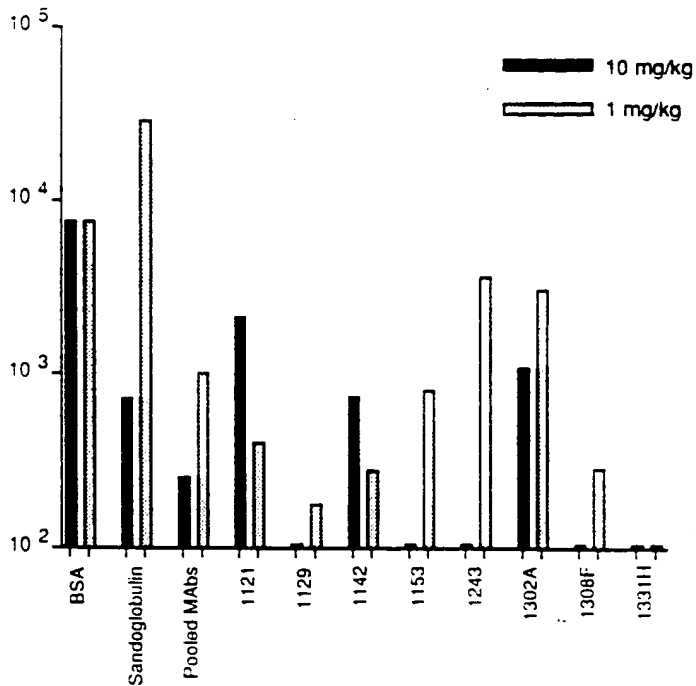


Figure 2  
Prophylaxis of RSV Infection in *S. Hispidus*  
Using Anti-F Monoclonal Antibodies

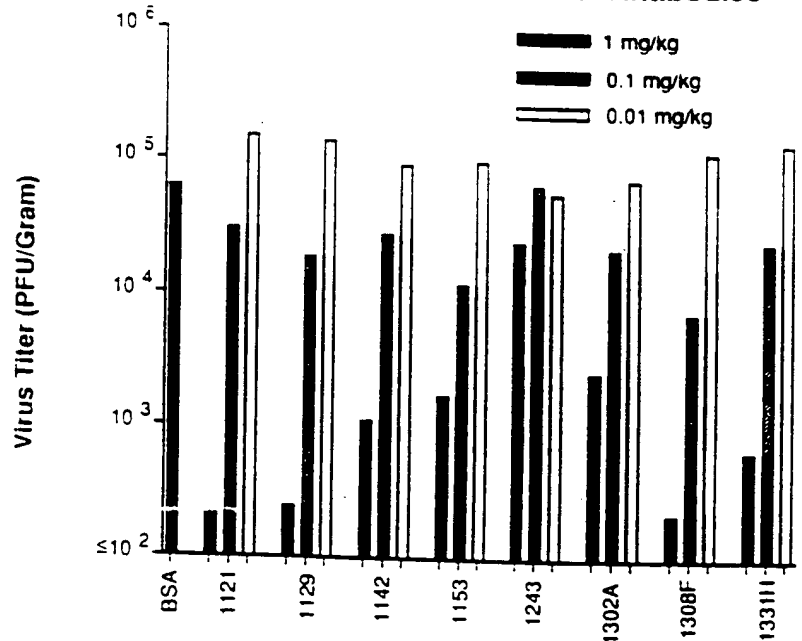


Figure 3

5 10 15 20 HumanHV3  
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Ser Val Lys Val  
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Ser Val Lys Val  
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Arg Pro Gly Ala Leu Val Lys Leu  
25 30 35 40  
Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr Tyr Ile Tyr Trp Val Arg Gln Ala  
Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr Tyr Ile Tyr Trp Val Lys Gln Arg  
45 50 55 60  
Pro Gly Gln Gly Leu Glu Trp Met Gly Ile Ile Asn Pro Ser Gly Gly Ser Thr Ser Tyr  
Pro Gly Gln Gly Leu Glu Trp Ile Gly Trp Ile Asp Pro Gln Asn Gly Asn Thr Val Phe  
Pro Gln Gln Gly Leu Glu Trp Ile Gly Trp Ile Asp Pro Gln Asn Gly Asn Thr Val Phe  
65 70 75 80  
Ala Gln Lys Phe Gln Gly Arg Val Thr Met Thr Arg Asn Thr Ser Thr Ser Thr Val Tyr  
Asp Pro Lys Phe Gln Gly Arg Val Thr Met Thr Arg Asp Thr Ser Thr Ser Thr Val Tyr  
Asp Pro Lys Phe Gln Gly Lys Ala Ser Ile Thr Ser Asp Thr Ser Ser Asn Thr Ala Tyr  
85 90 95 100  
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Tyr Tyr Gly  
Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Tyr Tyr Gly

Figure 4

10 20 30 40 50 60 70 80 90 100  
Mu 1308F Asp Ile Lys Met Thr Gln Ser Pro Ser Ser Met Tyr Val Ser Leu Gly Gly Arg  
GAC ATC AAG ATC ACC CAG TCT CCA TCT TCC ATG TAT GTT TCT CTA GGA GAG AGA  
Hum K102 Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser Ala Ser Val Gly Asp Arg  
GAC ATC CAG ATG ACC CAG TCT CCT TCC ACC CTG TCT GCA TCT GTA GGA GAC AGA  
20 30 40 50 60 70 80 90 100  
Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Asn Arg Tyr Leu Asn Trp Phe Gln Gln  
GTC ACT ATC ACT TGC AAG GCG AGT CAG GAC ATT AAT AGG TAT TTA AAC TGC TTC CAG CAG  
Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Ser Trp Leu Ala Trp Tyr Gln Gln  
GTC ACC ATC ACT TGC GCG GCG AGT CAG AGT ATT AGT ACC TGC TTG GCC TGC TAT CAG CAG  
40 50 60 70 80 90 100  
Lys Pro Gly Lys Ser Pro Lys Thr Leu Ile His Arg Ala Asn Arg Leu Val Asn Gly Val  
AAA CCA GGG AAA TCT CCT AAG ACC CTG ATC CAT CCT CCA AAC AGA TTG CTA CAT GGG GTC  
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Asp Ala Ser Ser Leu Glu Ser Gly Val  
AAA CCA GGG AAA GCG CCT AAG CTC CTG TAT GAT GAT TCC ACT TTG GAA AGT GGG GTC  
60 70 80 90 100  
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Gln Glu Tyr Ser Leu Thr Ile Ser Ser Leu  
CCA TCA AGG TTC AGT GCG AGT GGA TCT GCG CAA GAA TAT TCT CTC ACC ATC AGC AGC CTG  
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu  
CCA TCA AGG TTC AGC GCG AGT GGA TCT GCG ACA GAA TTC ACT CTC ACC ATC AGC AGC CTG  
80 90 100  
Glu Phe Glu Asp Met Gly Ile Tyr Tyr Cys Leu Gln Thr Ala Gln Phe Pro Tyr Thr Phe  
GAA TTT GAA GAT ATG GGA ATT TAT TAT TGT CTA CAG TTT CAT GAG TTT CCC TAC AGC TTT  
Gln Pro Asp Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ser Tyr Ser  
CAG CCT CAT GAT TTT GCA ACT TAT TAC TGC CAA CAG TAT AAT AGT TAT TCT  
100  
Gly Gly Gly Thr Lys Leu Glu Ile Lys  
GGA GGG GGG ACC AAG CTG GAA ATA AAA



PRINCIPAL INVESTIGATOR: Johnson, Leslie Sydnor

### CHECKLIST (Front)

This is the required last page of the application.  
Check the appropriate boxes and provide the information requested.

#### TYPE OF APPLICATION

☐ NEW application (This application is being submitted to the PHS for the first time.)

☒ REVISION of previously submitted application no. 1R43AI30300-01  
This application replaces a prior unfunded version of a new application.)

☒ Change of Principal Investigator (If applicable)  
Name of Former Principal Investigator Young, James Francis

#### ASSURANCES (See appendix to instructions.)

a. Civil Rights

☒ Filled

☐ Not Filled

b. Handicapped  
Individuals

☒ Filled

☐ Not Filled

c. Vertebrate Animals  
(if applicable)

☒ Filled

☐ Not Filled

d. Human Subjects  
(if applicable)

☒ Filled

☐ Not Filled

#### ADDITIONAL ASSURANCES

The following additional certifications described below are made by checking the appropriate boxes and verified by the signature of the OFFICIAL SIGNING FOR APPLICANT ORGANIZATION on the FACE PAGE of the application.

f. Delinquent Federal Debt. ☒ No ☐ Yes (If "Yes," attach explanation.)

Before a grant award can be made, the applicant organization must certify that it is not delinquent on the repayment of any Federal debt. The certification applies to the applicant organization, not to the person signing the application as the authorized representative nor to the principal investigator.

Examples of Federal debt include delinquent taxes, audit disallowances, guaranteed or direct student loans, FHA loans, business loans, and other miscellaneous administrative debts. For purposes of this certification, the following definitions of delinquency apply:

- For direct loans (whether awarded directly to the applicant by the Federal Government or by an institution using Federal funds), a debt more than 31 days past due on a scheduled payment.
- For guaranteed and insured loans, recipients of a loan guaranteed by the Federal Government that the Federal Government has repurchased from a lender because the borrower breached the loan agreement and is in default.
- For grants, organizations in receipt of "Notice of Grants Cost Disallowance" which have not repaid the disallowed amount or which have not resolved the disallowance. (Definition excludes cost disallowances in an "appeal" status.)

Where the applicant discloses delinquency on debt to the Federal Government, the PHS shall (1) take such information into account when determining whether the prospective grantee organization is responsible with respect to that grant, and (2) consider not making the grant until payment is made or satisfactory arrangements are made with the agency to whom the debt is owed. Therefore, it may be necessary for the PHS to contact the applicant before a grant can be made to confirm the status of the debt and ascertain the payment arrangements for its liquidation. Applicants that fail to liquidate indebtedness to the Federal Government in a businesslike manner place themselves at risk of not receiving financial assistance from the PHS.

g. Debarment and Suspension. ☒ No ☐ Yes (If "Yes," attach explanation.)

Before a grant award can be made, the applicant organization must certify, among other things, that neither it nor its principals are presently debarred, suspended, proposed for debarment, declared ineligible, or voluntarily excluded from covered transactions by any Federal department or agency. Subawardees, that is, other corporations, partnerships, or other legal entities (called "lower tier" participants), must make the same certification to the applicant organization concerning their covered transactions. Please refer to the pertinent DHHS implementing regulations, Title 45 Code of Federal Regulations Part 76, for complete certification requirements.

11

Exhibit 2

Project No. \_\_\_\_\_

TITLE \_\_\_\_\_

Book No. \_\_\_\_\_

From Page No. \_\_\_\_\_

Sequence (Triplets): CAC GTC GAC ATT CAG CTG ACC  
CAG TCT CCA SAT Pro II  
~~VER~~ general  $V_H$  5'  
 SJ41 conc. = 2.15 mg/ml

Sequence (Triplets): AGC GGATCC AGG GGC CAG TGG  
ATA GAC Bam HI  
 3' Mouse Gamma primer w BamHI site (Heavy chain)  
 SJ10 conc. = 1.247 mg/ml

Sequence (Triplets): CGG AAT TCA GGT TTA ICT GCA  
GIAGTC WGG RI Pst I  
 [A]  
 general  $V_H$  5' SJ42 conc. = 2.46 mg/ml

Sequence (Triplets): GAT GGA TCC AGT TGG TGC AGC  
ATC Bam  
 3' mouse kappa primer with BamHI site (light chain)  
 SJ11 conc. = 2.402 mg/ml

Witnessed & Understood by me,

*Robert L. Bennett*

Date

Invented by

*Robert L. Bennett*

Recorded by

Date

1/22/91

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J41 :

$$(330)(30) = 9900$$

$$(9900)^{-1} (2.15 \times 10^{-3} \text{ gm/ml}) = 2.17 \times 10^{-7} \text{ mol/ml} = 217 \text{ pmol/l}$$

$$(217)x = (25)(1000)$$

$$x = 115.2 \mu\text{l per ml}$$

J10 :

$$(330)(27) = 8910$$

$$(8910)^{-1} (1.247 \times 10^{-3}) = 1.40 \times 10^{-7} \text{ mol/ml} = 140 \text{ pmol/l}$$

$$140x = (25)(1000)$$

$$x = 178.6 \mu\text{l per ml}$$

J42 :

$$(330)(30) = 9900$$

$$(9900)^{-1} (2.66 \times 10^{-3} \text{ gm/ml}) = 2.69 \times 10^{-7} \text{ mol/ml} = 269 \text{ pmol/l}$$

$$269x = (25)(1000)$$

$$x = 92.9 \mu\text{l per ml}$$

J11 :

$$(330)(24) = 7920$$

$$(7920)^{-1} (2.4 \times 10^{-3}) = 3.03 \times 10^{-7} \text{ mol/ml} = 303 \text{ pmol/l}$$

$$303x = (25)(1000)$$

$$x = 82.5 \mu\text{l per ml}$$

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From Page No. 1-22-91 PCR Reaction to generate  $V_L$  and  $V_H$  fragments from A region Msh 112

Tube	Fragment	Template	Primers	Program
A	$V_L$ 1129 Bam-Sal	1129 cDNA	SJ41 SJ11	94°C 2 min 94°C 1 min 55°C 2 min 72°C 2 min
B	$V_H$ 1129 Eco-Bam	1129 cDNA	SJ42 SJ10	8°C soak o/n

1  $\mu$ L 1129 cDNA + 19  $\mu$ L  $H_2O$

10  $\mu$ L

10  $\mu$ L

4  $\mu$ L 5' primer

4  $\mu$ L 3' primer

10  $\mu$ L 10x PCR

10  $\mu$ L 10x dNTP

60  $\mu$ L  $H_2O$

1  $\mu$ L AmpiTaq

A B C



A-  $V_L$  1129

B-  $V_H$  1129

C- Sid's PCR reaction.

1-23-91

phenol/chloroform ext, chloroform ext, ethanol ppt, ethanol wash, resuspend in 50  $\mu$ L TE

digestion  $V_L$  1129 PCR fragment: 50  $\mu$ L DNA

4  $\mu$ L React 3

2  $\mu$ L SalI

2  $\mu$ L BamHI

incubate 1.0 hr at 37°C

digestion at  $V_H$  1129 PCR fragment

50  $\mu$ L DNA

4  $\mu$ L React 3

2  $\mu$ L EcoRI

2  $\mu$ L BamHI

incubate 1 hr at 37°C

digestion of pUC18: 10  $\mu$ L DNA

3  $\mu$ L TE

4  $\mu$ L React 3

1  $\mu$ L enzyme: BamHI + EcoRI

or BamHI + SalI

10  $\mu$ L

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om Page No. 3 Ligation Reactions:

	puc18 Sal-Bam control	puc18 EcoRI-Bam control	puc18 V <sub>H</sub> 1129	puc18 H <sub>H</sub> 1129
UC18 Sal-BamHI f.	3 $\mu$ l	-	3 $\mu$ l	-
UC18 EcoRI-BamHI f.	-	3 $\mu$ l	-	3 $\mu$ l
V <sub>H</sub> 1129 Sal-BamHI f.	-	-	7.5 $\mu$ l	-
H <sub>H</sub> 1129 EcoRI-BamHI f.	-	-	-	7.5 $\mu$ l
E.	12 $\mu$ l	12 $\mu$ l	4.5 $\mu$ l	4.5 $\mu$ l
ligase Buffer	4 $\mu$ l	4 $\mu$ l	4 $\mu$ l	4 $\mu$ l
ligase	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l

incubate O/N at 16°C

-25-91

Transformed ligation mixtures into DH5 $\alpha$  and plated on SB LBamp/X-gal

Results:

ligation	# colonies
puc18 Sal-Bam	6 blue / 2 white
V <sub>H</sub> 1129	3 blue / 400 white
puc18 Eco-Bam	3 blue / 1 white
V <sub>H</sub> 1129	7 blue / 68 white

picked 6 V<sub>H</sub>1129 colonies and 6 V<sub>H</sub>1129 colonies and grew in LBamp (O/N in refrig.)  
 (1-6) (7-12)

1-29-91



← puc18  
 ← hopefully supercoiled  
 ← V<sub>H</sub>1129

• will digest with less DNA or for longer time in order to see if supercoiled band disappears

• will load more DNA of puc18 V<sub>H</sub>1129 to see if clones 1,2,3,4,6 have insert.

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From Page No. 1-30-91

### Miniprep plasmid DNA sequencing:

- After 1ml ethanol pption, dry pellet, resuspend in 100µl TE plus 100µg/ml RNase A
- Incubated @ 37°C for 30'
- phenol/chloroform extracted
- Added 35µl 20% PEG, 2.5M NaCl
- 30' on ice
- spin 5'
- wash w 70% ethanol, vac dry 2X
- resuspended in 25µl TE

Use 2µg DNA template in 8µl volume

Add 2µl 2M NaOH

Incubate at RT for 5 min

Add 3µl 3M NaOAc pH 4.5

2µl H<sub>2</sub>O

5µl primer at 4pmoles/µl (10µg/µl)

(GTAAACGACGACCACT)

5µl Sequencing Primer (Universal) (0.5pmo

5µl SJ2 (reverse primer) 10µg/µl

75µl for 5min, rinse w 70% ethanol 2X, vac dry, resuspend in 25µl TE

100% ethanol

### Annealing

2µg DNA 8µl of DNA/primer mix

2µl Sequencing buffer

Anneal by heating 2min at 65°C, then cooled slowly to 235°C

While cooling fill and cap tubes w 2.5µl of ea. termination mixture

Dilute labeling mix 1:5 to working conc. if needed.

Dilute Sequenase for all templates in ice cold TE buffer

Pre-warm 4 termination tubes in 37°C bath

8µl: 32µl H<sub>2</sub>O

5µl: 35µl Exz. Dil.

LABELING RXN: To 10µl annealed DNA mixture (1µl)

0.1M DTT 1µl

Diluted labeling Mix 2µl

[<sup>35</sup>S] dATP 0.5µl

Diluted Sequenase 2µl

incubate at RT, 5 min

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on Page No. 5TERMINATION RXN

Transfer 3.5  $\mu$ l of labeling rxn to each termination tube, mix and continue incubation of the term rxns at 37°C for 5 min

Stop rxn by adding 4  $\mu$ l Stop Soln

Heat samples to 75°C for 2 min prior to loading

Gel mix: 42 g urea

5.8 g acrylamide

0.2 g bis

0.1 g APS

10 ml 10x TBS

100 ml total volume

loading order:

V <sub>L</sub> 1129 *1 f.	V <sub>H</sub> 1129 7 f.	-76-9 r	#25
V <sub>L</sub> 1129 2 f.	V <sub>H</sub> 1129 10 f.	-76-10 r	#16
V <sub>L</sub> 1129 5 f.	V <sub>H</sub> 1129 7 r.	-76-11 r	
V <sub>L</sub> 1129 1 r.	V <sub>H</sub> 1129 10 r.	-76-12 r	
V <sub>L</sub> 1129 2 r.	✓ V <sub>H</sub> 1129 12 r.		
V <sub>L</sub> 1129 5 r.			

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6% Sequencing gel: 76-9 V<sub>H</sub> 1129 # 7 r  
 76-10 8 r  
 76-11 9 r  
 76-12 10 r  
 V<sub>H</sub> 1129 # 7 f 11 r  
 V<sub>H</sub> 1129 # 8 f 12 r  
 10 f  
 11 f  
 12 f

Primers: forward primer - 40 primer supplied by sequenase kit  
 reverse primer S112

Result: all V<sub>H</sub> 1129 clones sequenced are the incorrect heavy chain MOPC.  
 all V<sub>L</sub> 1129 clones sequenced appear to be the correct sequence

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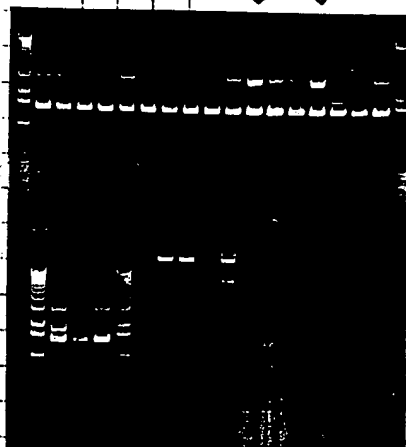
2-11-91

• Picked 20 pUC18 V<sub>H</sub> 1129 clones from original transformation

• Analyzed each plasmid by digestion w/ Xba I

• V<sub>H</sub> 1129 contains Xba I site

MOPC21 does not contain Xba I site



1-20: pUC18 V<sub>H</sub> 1129 Xba I

#10 uncut

#20 uncut

#11 and #14 appear to be the only correct clones

→ sequence 11, 14 (12 as negative)

Grow #11 and #14 up as midi prep

### 3 Sequencing ran:

1- pUC18 V<sub>H</sub> 1129 #11 (S112) reverse primer ←

2- pUC18 V<sub>H</sub> 1129 #12 (S112)

3- " #14 (S112)

4- pUC18 V<sub>H</sub> 1129 #15 (S112) ←

5- " #12 "

6- " #14 "

7-

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11

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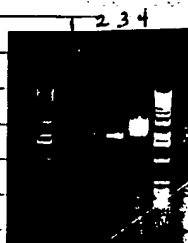
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From Page No. 2/18/91

- Grew 2 ml cultures of 75+76 #1-3 to ~mid-log
- Infected with F10 by adding 10  $\mu$ l to 2 ml cultures
- Grew for 5 hrs.
- Spun down and saved supernatant in refrigerator

2-19-91

- Precipitated phage by adding equal volume 13% PEG 1.5M NaCl
- Ice for 30 min
- Spun down for 15 min at 10,000 rpm
- Resuspended in 300  $\mu$ l TE
- phenol/chloroform ext., chloroform ext., ethanol ppt, ethanol wash, c
- Resuspended in 20  $\mu$ l TE



$0.2 \times 10 = 2 \mu\text{g}$

1-3) 75+76 (1-3)

4) ssDNA control from sequenase kit



1- V<sub>H</sub> 1129 #14 BamHI-EcoRI

2- V<sub>H</sub> 1129 #105 BamHI-Sal

3- V<sub>H</sub> 1129 #111 BamHI-EcoRI



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Prep for sequencing  $V_{H1129}$  #11, #14,  $V_{L1129}$  #5 midis; 75+76 #1, #2, #3 :

$V_{H1129}$  #11 } RNased, phenol/chloroform extraction  
 $V_{H1129}$  #14 } PEG ppted 20% PEG, 2.5M NaCl  
 $V_{L1129}$  #5 } ethanol washed 2x, dried  
 resuspended in 20  $\mu$ l TE

removed 8  $\mu$ l DNA

added 2  $\mu$ l 2M NaOH, incubated at RT for 5 min.

added 3  $\mu$ l NaOAc pH 5.2, 2  $\mu$ l H<sub>2</sub>O, 5  $\mu$ l primer at 4  $\mu$ mol/ $\mu$ l (10  $\mu$ g/ $\mu$ l) (S12)

added 75  $\mu$ l ethanol, spin 5 min, wash with 70% ethanol, dry  
 resuspend in 8  $\mu$ l TE

Sequencing gel order

- 1 75+76 #1 r
- 2 75+76 #2 r
- 3 75+76 #3 r
- 4  $V_{L1129}$  r
- 5  $V_{H1129}$  r 11
- 6  $V_{H1129}$  r 14

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LBennett

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Date

2/20/91

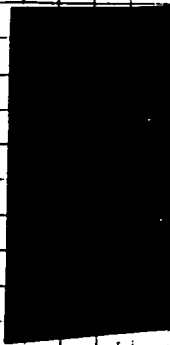
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PSH1 HindIII - Pst 36ul DNA  
 4ul React 2  
 2ul HindIII  
 8ul Pst I  
 1.5 hr incubation  
 gel fractionate  
 Gene Clean  
 resuspend in 20ul TE

Kinase treatment: ~~PSH1~~ Pst-HindIII 12ul  
 2ul 10X glycerol-kinase buffer 2ul  
 2ul 10mM ATP 2ul  
 0.1M DTT 1ul  
 T4 Kinase 1ul  
 10ul

incubate at 37°C > 1hr. (too long)

Annealed S1101 (1ul) + S1102 (1ul) + 2ul Rxn Buffer + 1ul H<sub>2</sub>O  
 65°C 3min, slow cooled

Ligation reaction: 10ul PSH1 Pst-HindIII (kinased)  
 10ul annealed linker  
 4ul Ligase Buffer  
 1.5ul Ligase  
 O/N incubation

RESULT: PSH1 is the wrong vector to be using for this experiment. Repeat w/ PSH.

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PCR Reaction

Tube	Template	Primers	Fragment	Program
3	pUC18 V <sub>L</sub> 1129#5 mini 1 µl	SJ103 (10 µg) SJ104 (10 µg) 1 µl	EcoRV-HindIII V <sub>L</sub> 1129	2 min 94°C 1 min 94°C 2 min 60°C 2 min 72°C } 30x 8°C soak o/N

22

1	pUC18 V <sub>L</sub> 1129 1 µl	<del>SJ103</del> (25 pmol/l) SJ104	V <sub>L</sub> 1129 EcoRV-HindIII	2 min 94°C 1 min 94°C 2 min 48°C 2 min 72°C } 30x 8°C soak o/N
2	pUC18 H <sub>V</sub> 1129 1 µl	SJ105 SJ106	V <sub>H</sub> 1129 f.	
3	CDNA 1129 1 µl	SJ105 SJ106	V <sub>H</sub> 1129 f.	

one by Sid 2/27/91

	Template	Primers	Fragment	Program
1	CDNA 1129	SJ105 SJ106	V <sub>H</sub> 1129	1 min 94°C 2 min 57°C 2 min 72°C
2	pUC18 V <sub>L</sub> 1129	SJ105 SJ106	V <sub>L</sub> 1129	

Note: use more Taq polymerase

Reviewed &amp; Understood by me.

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Invented by

R. Schmidt

Recorded by

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From Page No. 2/22/91

SJ103 - 1129 and general VL 5' EcoRI, ClaI, EcoRV

$$\text{gCg} \overbrace{\text{gAA TTC AgA TCg ATA TCC AAC TgA CCC AgT CTC CA}}^{\text{EcoRI} \quad \text{ClaI}}$$
STOCK CONC.  $1.0 \text{ mg/ml}$  EcoRI

$$(330)(38) = 12,540$$

$$(12,540)^{-1} (1 \times 10^{-3} \text{ g/ml}) = 8.0 \times 10^{-8} \text{ mol/ml} = 80 \text{ pmol/l}$$

$$80x = (25)(100)$$

$$x = 31.25 \text{ } \mu\text{l} \text{ in } 100 \mu\text{l H}_2\text{O}$$

SJ104 - 1129 VL 3' HindIII

TAT TTC AAg CTT ggT CCC CTA gCC gAA CgT

$$(330)(30) = 9900$$

$$(9900)^{-1} (1 \times 10^{-3}) = 1.0 \times 10^{-7} = 100 \text{ pmol/l}$$

$$100x = (25)(100)$$

$$x = 25 \mu\text{l} \text{ in } 100 \mu\text{l H}_2\text{O}$$

SJ105 - 1129 VH 5'

$$\text{ggT TCT ggT AAA TCT TCC gAA ggT ggT AAA ggT CAg gTg CAg CTg}$$

$$\text{CAg CAg TC(A,T) gg}$$

$$(330)(56) = 18,480$$

$$(18,480)^{-1} (1 \times 10^{-3}) = 5.4 \times 10^{-8} = 54 \text{ pmol/l}$$

$$54x = (25)(100)$$

$$x = 46.3 \mu\text{l} \text{ per } 100 \mu\text{l H}_2\text{O}$$

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SJ106 - 1129, 98-6 VH 3'

~~gCg Agg~~ <sup>BamHI</sup>

gCg Agg ATC CTT ATg AgC TCA Cgg TgA C

$$(330)(28) = 9240$$

$$(9240)^{-1} (1 \times 10^{-3}) = 1.1 \times 10^{-7} = 110 \text{ pmol}/\lambda$$

$$(110)x = (25)(100)$$

$$x = 23 \mu\text{l per } 100 \mu\text{l H}_2\text{O}$$

SJ108 - H1308 3' linker

<sup>HindIII</sup>

Cgg AAg ATT TAC CAg AAC CAg Agg Tgg ACC CTT TTA TTT CAA gCT

Tgg TCCC

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Invented by

Libermann

Recorded by \_\_\_\_\_

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2/22/71

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From Page No. 2/23/11

Western blot to test J212-MB-KLH rabbit #1 antibody:

- |                       |  |
|-----------------------|--|
| 1- Low MW Marker std. | 4-20% gradient gel                                 |
| 2- 100ng 4-4-20/212   | transferred to nitrocellulose 1 hr                 |
| 3- 500ng 4-4-20/212   | cut into three segments                            |
| 4- Low MW Marker std. | blot blocked 30 min with milk                      |
| 5- 100ng 4-4-20/212   | blotted with 1° Ab at 1/200, 1/500, 1/250 dilution |
| 6- 500ng 4-4-20/212   | washed   |
| 7- Low MW Marker      | blotted with 2° Ab at 1/3000 1 hr                  |
| 8- 100ng 4-4-20/212   | washed   |
| 9- 500ng 4-4-20/212   | developed with ECL                                 |
| 10-                   |  |

1° Ab = J212-MB-KLH (rabbit) (test bleed)

2° Ab = goat-anti-rabbit 1/3000 IgG HRP

See blot notebook for results: Antibody reacts to protein at 1/250 dilution

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Invested by

*A. Barnett*

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From Page No. 2/28/91

WESTERN BLOT to determine titer of J212-MB-KLH

4 20% SDS-PAGE

1- Low MW	1- Low MW
2- 50ng 4-4-20/212	2- 50ng 4-4-20/212
3- Low MW	3- Low MW
4- 50ng 4-4-20/212	4- 50ng
5- Low MW	5- Low MW
6- 50ng 4-4-20/212	6- 50ng
7- Low MW	7- Biorad MW Std.
8- 50ng 4-4-20/212	8- G-98
9- Low MW Std.	9- G-126
10- 50ng 4-4-20/212	10-

Transferred to nitrocellulose 1 hr at 350mAmp

Blocked in 5% Milk, O/N at RT

Blotted with J212-MB-KLH #1 & #2 Test bleeds at  $1/1000$ ,  $1/5000$ ,  $1/12500$  } 4hr. at RT

Blotted one strip with pre-bleed sera from rabbit #1 at  $1/1000$

Witnessed &amp; Understood by me.

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J. Bennett

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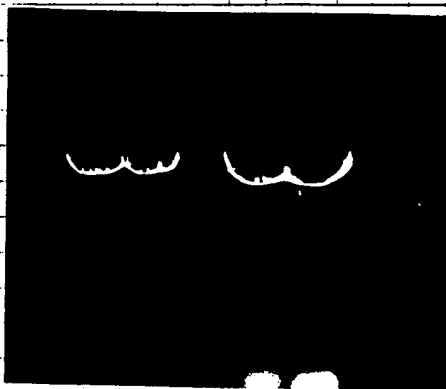
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From Page No. 3/1/91

Digested pS18 w HindIII + Pst I : 3µl pS18  
 4µl React 2  
 2µl HindIII  
 2µl Pst I

Digested pS18 w HindIII : 5µl pS18 5µl H<sub>2</sub>O  
 1.3µl react 2 1.3µl React  
 1.0µl HindIII 1.0µl Pst I

- 1- pS18 HindIII
- 2- pS18 Pst I
- 3- pS18 Pst I + HindIII
- 4- pS18 Pst I + HindIII



Isolated the <sup>3-4</sup> fragments and gene cleaned - resuspended in 40µl

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for the generation of  $V_L + V_H$  1129 by overlapping PCR:

$V_L$  1129 PCR fragment (from pg. 14): phenol/chloroform  
 chloroform  
 ethanol ppt  
 gel purify & Gene Clean

$V_H$  1129 PCR fragment: Sid cleaned  
 gel purified & Gene Cleaned

Preparative gel

1-  $V_L$  11292-  $V_H$  1129

PCR Reaction (overlapping PCR)

Tube	Fragment	Template	primers	Program
1	$V_L - V_H$ 1129	$V_L f + V_H f$ 1129	S1103 S1106	94°C 2 min 94°C 1 min 55°C 2 min } 30X 72°C 2 min 8°C soak

RESULT: NO AMPLIFICATION

 $V_L - V_H$  1129 $V_L f + V_H f$  11293 $\mu$ l 3 $\mu$ l

S1103

S1106

94°C 2 min

94°C 1 min

45°C 2 min

72°C 2.5 min

94°C 1 min

55°C 2 min

72°C 2.5 min

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J. H. Smith

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From Page No. 19

3/4

Kinase treatment of pS18 Pst-HindIII fragment :

15  $\mu$ l pS18 Pst-HindIII f.  
2  $\mu$ l 10X Ligase buffer  
2  $\mu$ l 10mM ATP  
1  $\mu$ l T4 Kinase  
20  $\mu$ l total volume  
45 min at 37°C  
heat inactivated at 65°C 10 min

Annealed S1101 and S1102 :

1  $\mu$ l S1101 (1mg/ml)  
1  $\mu$ l S1102 (1mg/ $\mu$ l)  
5  $\mu$ l H<sub>2</sub>O  
heated to 65°C 2 min  
slow cooled to < 37°C

Mixed 10  $\mu$ l pS18 + 3  $\mu$ l annealed S1101-S1102 + 1  $\mu$ l ~~to~~ ligase  
incubated at 16°C for 2 hrs.

Transformed into HB101 (why not DH5- $\alpha$ ?)

Result: 1 white transformant

picked white colony and grew as mini prep.

To Page

Witnessed & Understood by me.

Date

Invented by

ABlenitt

Date

3/9/91

Recorded by

1 1 1  
TITLE

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

From Page No. 3/4/91

PCR to generate  $V_L - V_H$  1129

Tube	Fragment	Primers	Template	Program
1	<del>puc18 V<sub>L</sub> 1129</del> $V_L$ 1129	SJ103 SJ104	puc18 $V_L$ 1129	2 min 94°C 1 min 94°C 2 min 48°C 2 min 72°C } 30X
2	$V_H$ 1129	SJ105 SJ106	puc18 $V_H$ 1129	2 min 94°C 1 min 94°C 2 min 42°C 2 min 72°C } 10X 1 min 94°C 2 min 50°C 2 min 72°C 8°C soak
3	$V_L - V_H$ 1129	SJ103 SJ106	$V_L$ 1129 f $V_H$ 1129 f	"
	$V_H$ 1129 $V_L$ 1129			

Overlapping PCR did not work because the overlap is too small.  
Need to do another PCR reaction to extend the overlap on  $V_L$  1129

3/5/91

Tube	Fragment	Primers	Template	Program
1	$V_L$ 1129	SJ103 (1 $\mu$ l) (new) SJ105 (1 $\mu$ l 25 $\mu$ mol) (from above)	$V_L$ 1129 PCR 1 $\mu$ l (from above)	2 min 94°C 1 min 94°C 2 min 50°C 2 min 72°C } 30X 8°C soak

Note:  $V_L$  1129 PCR is not cleaned at all; used straight from PCR reaction.

To Page

Witnessed & Understood by me,

Date

Invented by

*Debra J. Bennett*

Recorded by

Date

3/5/91

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_ TITLE \_\_\_\_\_

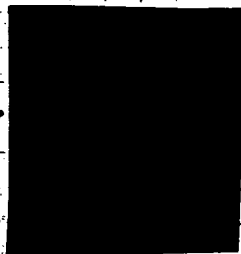
Page No. 23Overlapping PCR to generate  $V_L 1129 - V_H 1129$ 

Tube	Fragment	Template	Primers	Program
4(1)	$V_L - V_H 1129$	$V_L 1129 103-108$	SJ103	94°C 2min
		purified $V_H 1129 105-106$	SJ106	94°C 1min
				45°C 2min } 30X
				72°C 2min
				8°C soak



← expecting a ~750bp fragment

3/7	Fragment	Template	Primers	Program
2	$V_L - V_H 1129$	$V_L 1129 103-108$ (1 $\mu$ l)	SJ103	94°C 2min
		purified $V_H 1129 105-106$ (1 $\mu$ l)	SJ106	94°C 1min
3	$V_L - V_H 1129$	SJ103-SJ106 (above PCR)	SJ103	55°C 2min } 30X
			SJ106	72°C 2min
				8°C soak



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by

Dwight Bennett

Recorded by \_\_\_\_\_

Date

3/7/91

1 1 1

Project No. \_\_\_\_\_

TITLE \_\_\_\_\_

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From Page No. \_\_\_\_\_

3/6/91



1- pCMVPA 5µl  
2- 2627 5µl (Sid vector)  
3- 5410 5µl (Sid vector)

Digested 10µl 5410	14µl 2627	20µl pCMVPA
26µl H <sub>2</sub> O	22µl H <sub>2</sub> O	16µl H <sub>2</sub> O
4µl React 3	4µl React 1	4µl React 1
2µl BamHI	1µl Cla	1µl Cla
2µl BglI	1hr digestion	1hr digestion
1hr digestion		
	add 0.4µl 3M NaCl	add 0.4µl 3M NaCl
BglI →	2µl BglI	2µl BamHI
	incubate 1 hr.	incubate 1 hr.

Fractionate on 17% preparative gel  
Isolate fragments and clean w Gene Clean

3/7/91

Redigestion of 5410 and 2627 w BglI this time:

10µl 5410	14µl 2627
26µl H <sub>2</sub> O	22µl H <sub>2</sub> O
4µl React 2	4µl React 2
2µl BamHI	2µl BglI
2µl BglI	2µl ClaI
1hr incubation at 37°C	1hr incubation at 37°C

Prep.

Gel: 1- 5410 BamHI

2- 2627 BglI

Isolated pCMVPA 1000kb ClaI-BamHI f.

Isolated 3000kb 5410 BamHI-BglI f.

Isolated 1000kb 2627 ClaI-BglI f.

Gene Cleaned

To Page

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3/7/91

From Page No. 25Resuspended  $\lambda$  fragment in 5  $\mu$ l TE.Mixed fragments for ligation: 15  $\mu$ l DNA2  $\mu$ l Ligase Buffer (10X)2  $\mu$ l 10mM ATP1  $\mu$ l Ligase

Incubated O/N at 16°C

8/91

Transformed into DH5- $\alpha$  and plated on LB Amp media.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

D. J. Bennett

Date

3/8/91

Recorded by



111

Project No. \_\_\_\_\_

TITLE \_\_\_\_\_

Book No. \_\_\_\_\_

From Page No. 3/6/91

SDS-PAGE GEL (for Sid) 4-20% Gradient Gel

- no apparent expression on this Western

Sid has the blots -

1- 4-4-20 std. 50 ~~mg~~

2- super. #1

3- #2

4- #3

5- #4

6- 1 MW std

7- pellet #1

8- #2

9- #3

10- #4

Transferred to nitrocellulose

Blocked w 5% milk for 30 min

Blotted w 1° Ab (rabbit 1212-MB-KUH #1) 1/2000 for 30 min

Washed

Blotted w 2° Ab (goat anti-rabbit IgG-HRP) 1/3000 for 30 min

Washed

Developed with <sup>detection</sup> ECL System

To Page 1

Witnessed &amp; Understood by me: \_\_\_\_\_

Date \_\_\_\_\_

Initiated by

\_\_\_\_\_  
Recorded by

Date

3/6/91

From Page No. 23

3-8-91

- Cleaned PCR fragment : phenol/chloroform ext., chloroform ext., ethanol ppt, ethanol u

- Digested w EcoRI-BamHI : 36ul V<sub>L</sub>-V<sub>H</sub> 1129 Acc f. (resuspended in TE)  
4ul Restr 3  
2ul EcoRI  
2ul BamHI  
Incubate 1 hr at 37°C

- Digested pUC18 w EcoRI + BamHI : 10ul pUC18

26ul TE

4ul Restr 3

2ul EcoRI

2ul BamHI

Incubate 1 hr. at 37°C

Preparative Gel to isolate V<sub>L</sub>-V<sub>H</sub> 1129 750 bp band & pUC18 Eco-Bam

1 2

1-V<sub>L</sub>-V<sub>H</sub> 1129 EcoRI-BamHI

2 pUC18 EcoRI-BamHI

Isolated these fragments, Gene Cleaned  
resuspended V<sub>L</sub>-V<sub>H</sub> 1129 in 10ul

pUC18 Eco-BamHI in 20ul

## Ligation Reaction

10ul V<sub>L</sub>-V<sub>H</sub> 1129 EcoRI-BamHI

5ul pUC18 EcoRI-BamHI

2ul 10X Ligase Buf.

2ul 10mM dATP

1ul ligase

Incubated ~~16~~ 16°C for 45 min

Transformed into DH5-α, outgrow 1 hr. plate on LB Amp

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Investigator by

J. J. Bennett

Date  
3/8/91

Recorded by

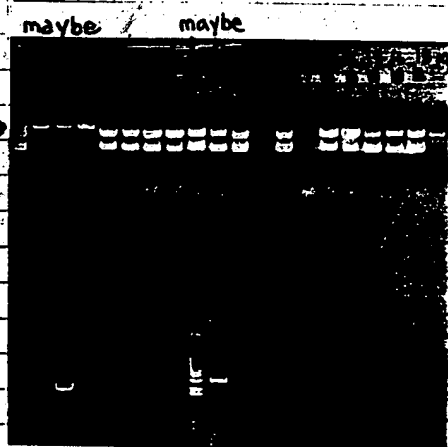
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Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

From Page No. <sup>24</sup> 3-11-91

• Started miniprep to screen 5410-2627-pCMVPA (1-3) }  
puc18 V<sub>L</sub>-V<sub>H</sub> (4-18) } 2ml in LBamp  
puc18 (19-20) }

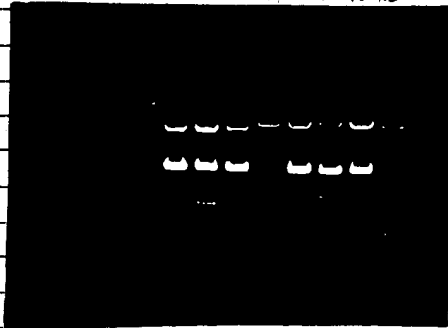
screen 5410-2627-pCMVPA (1-3) w EcoRI → to linear form ~ 5000 kb  
screen puc18 V<sub>L</sub>-V<sub>H</sub> (4-18) w EcoRI  
puc18 (19-20) w EcoRI  
1-15, 17, 18, 18-20



1-3) appear to be the correct size (5000)  
need to cut with enzyme from the l<sub>u</sub>  
HindIII, Sma or Sal

4-18) puc18 V<sub>L</sub>-V<sub>H</sub> transformation  
#5, #8, #9 may contain insert  
2.7kb puc18 band appears to be sl  
larger.  
Additional screening: EcoRI + B  
(to cut out insert)  
Also digest w/ HindIII

1 2 3 4 5 6 7 8 9 10 11 12 13



1- #1 BglI-SalI ✓  
2- #2 BglI-SalI ✓ Grow #1, 2, 3, 5, 8, 9  
3- #3 BglI-SalI ✓ midipreps. (all in l<sub>u</sub>)  
4- 5410 BglI-SalI ✓  
5- 2627 BglI-SalI ✓  
6- #5 EcoRI-BamHI ✓  
7- #8 EcoRI-BamHI ✓  
8- #9 EcoRI-BamHI -  
9- #20 EcoRI-BamHI ✓  
10- #5 HindIII ✓  
11- #9 HindIII  
12- #8 HindIII ✓  
13- #20 HindIII ✓

NOTE: Oligos SJ110-SJ118 were received today, 3-11-91.

Witnessed & Understood by me.	Date	Invented by <i>[Signature]</i>	Date 3/11/91
		Recorded by	

To Page

From Page No. 3-11-91

PCR to generate more V<sub>H</sub> 1129 105-106:

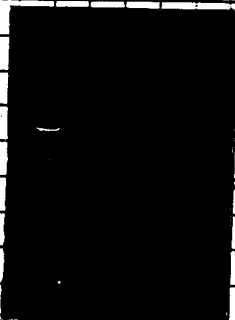
Tube	Fragment	Template	Primers	Program
11	V <sub>H</sub> 1129 105-106	pUC18 V <sub>H</sub> 1129 #11	SJ105 (fresh from stock)	94°C 2min
14		#14	SJ106 (25 pmol/μl)	94°C 1min
				46°C 2min } 10X
				72°C 2.5min
				94°C 1min
				55°C 2min } 20X
				72°C 2min
				8°C 50+K

Preparative gel  
11/14

← Isolated this 400 bp band to GeneClean

3-12-91

Template Fragment	Fragment	Primers	Program
V <sub>H</sub> 1129 103-108	V <sub>H</sub> 1129	SJ103 (fresh 25 pmol/μl)	94°C 2min
V <sub>H</sub> 1129 105-106		SJ106 (yesterday)	94°C 1min
			42°C 2min } 10X
			72°C 2min
			94°C 1min
			55°C 2min } 20X
			72°C 2min
			8°C 50+K



← Isolate this fragment

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Date

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Date

3/12/91

To Page No.

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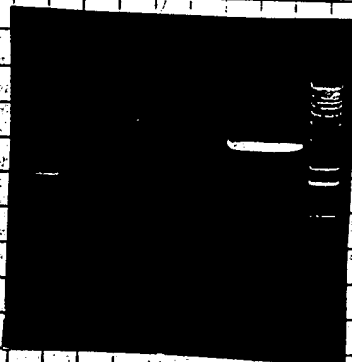
Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

From Page No. 29

- Peeled 5410-2627-pCMVPA clones 1,2,3 (minipreps)  
 Brought volume up to 36ul.  
 Digested with HindIII and salt

- Brought volume of pUC18 #2 miniprep to 36ul.  
 Digested with HindIII - EcoRI



1- 5410-2627 pCMVPA Cla + BamHI #1

2- #2

3- #3

4- 2627 Cla - BamHI

5- 5410-2627 pCMVPA BglI + BamHI #1

6- #2

7- #3

8- 2627 BglI - BamHI

9- 5410-2627 pCMVPA Cla + BglI #1

10- #2

11- #3

12- 2627 Cla + BglI

13- pUC18 V<sub>6</sub>-V<sub>11</sub> 1129 #5 EcoRI + BamHI

14- #8

15- #9

16- pUC18 EcoRI + BamHI

17- pUC18 V<sub>6</sub>-V<sub>11</sub> 1129 #5 HindIII

18- #8 HindIII

19- #9 HindIII

20- pUC18 #20 HindIII

To Page N

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Invented by \_\_\_\_\_

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Date \_\_\_\_\_

5/11/91

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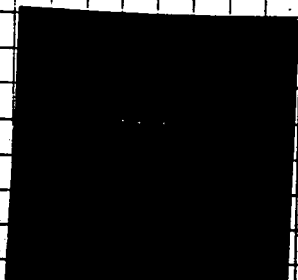
Project No. \_\_\_\_\_  
 B x No. \_\_\_\_\_

From Page No. 31 3-15-91

Construction of chimeric expression vector

36 $\mu$ l 2627	18 $\mu$ l 5401	5 $\mu$ l pCMVPA
4 $\mu$ l React 2	2 $\mu$ l React 2	13 $\mu$ l H <sub>2</sub> O
2 $\mu$ l ClaI	1 $\mu$ l BamHI	2 $\mu$ l React 1
2 $\mu$ l BglII	1 $\mu$ l BglII	1 $\mu$ l ClaI
		1 $\mu$ l BamHI

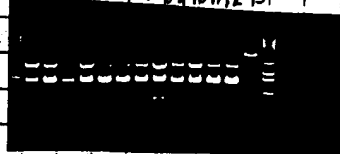
- 1- 2627 ClaI-BglII
- 2- 5401 Bam-BglII
- 3- pCMVPA Cla-BamHI



Isolated the fragments (circled) and GeneCleaned in one tube  
 Resuspended in 1x ligase buffer (17  $\mu$ l)  
 Ligated in 20  $\mu$ l total volume at 4°C  
 Six transformed into E. coli

Result —

1 2 3 4 5 6 7 8 9 10 11 12 13



1-112) 5401-2627 pCMV Bam-EcoRI ← wrong, looks like pUC  
 B) 2627 Bam-EcoRI

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3/15/91

To Page No. \_\_\_\_\_

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From Page No. \_\_\_\_\_

1. pUC18 digested w/ EcoRI and HindIII

Fragment purified and GeneCleared.

annealed primer oligos S1117 and S1118 1 µl of ea in 20 µl volume.  
slow cooled.

Ligation mixture: 5 µl pUC18 EcoRI + HindIII

1 µl S1117 + S1118

2 µl 10X ligase buf

2 µl dATP

10 µl H<sub>2</sub>O

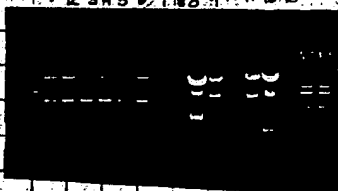
1 µl Ligase

Result: ~ 250-300 colonies  
(100 µl of cells)

~ 2-4 blue colonies

Transformed into DH5-α; plated on LBamp<sup>r</sup> + X-gal.

3-20-91



3-21-91

- started 50ml culture of pUC18 Neo-Not #1, 2, 3
- sequencing both of these, also see pg. 37

1- pUC18 link #1 NcoI

2- " #2 NcoI

3- " #3 NcoI

4- " #4 NcoI

5- " #5 NcoI

6- " #6 NcoI

7- pUC18 NcoI

8- pUC18 V<sub>1</sub>-V<sub>2</sub> 1129 #5 EcoRI-BamHI

9- " #9 EcoRI-BamHI

10- pUC18 EcoRI-Bam

11- pUC18 V<sub>1</sub>-V<sub>2</sub> 1129 #5 HindIII

12- " #9 HindIII

13- pUC18 HindIII

To Page No. \_\_\_\_\_

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3/20/91

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Project No. \_\_\_\_\_

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From Page No. 3-21-91

Sequencing of pUC18 V<sub>L</sub>-V<sub>H</sub> #5 & #9, p5401-2627-CMV #4, pUC18, pUC18 Neo-Not 1, 3, 75+76

5

Followed procedure on pg 5:

Reactions:

- 1- pUC18 V<sub>L</sub>-V<sub>H</sub> #5 midi forward primer SJ13 ✓
- 2- pUC18 V<sub>L</sub>-V<sub>H</sub> #5 midi SJ112 ✓
- 3- #5 midi SJ112; reverse primer ✓
- 4- pUC18 V<sub>L</sub>-V<sub>H</sub> #9 midi SJ13 ✓
- 5- pUC18 V<sub>L</sub>-V<sub>H</sub> #9 midi SJ112 ✓
- 6- pUC18 V<sub>L</sub>-V<sub>H</sub> #9 midi SJ12 ✓
- 7- p5401-2627-pCMV #4 mini SJ13
- 8- pUC18 midi SJ1-40
- 9- pUC18 Neo-Not #1 mini -40
- 10- pUC18 Neo-Not #3 mini -40
- 11- 75+76 #1 mini SJ12 } not correct
- 12- 75+76 #2 mini SJ12 } do not contain the desired mutant

PEG pptd (13% PEG, 1.5M NaCl) pUC18 V<sub>L</sub>-V<sub>H</sub> #9  
 p5401-2627-pCMV  
 pUC18  
 pUC18 Neo-Not 1 & 3  
 75+76 1, 2, 3

conc.	A <sub>260</sub>	dilution	extinction coeff.
pUC18 V <sub>L</sub> -V <sub>H</sub> #5	0.51	200	102 x 50 = 5100 µg/ml = 5.1 µg/µl
pUC18 V <sub>L</sub> -V <sub>H</sub> #9	0.263	200	52.6 x 50 = 2630 µg/ml = 2.63 µg/µl
pUC18	0.47	200	94 x 50 = 4700 µg/ml = 4.7 µg/µl

To Page No. \_\_\_\_\_

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Recorded by

Date 3/21/91



111

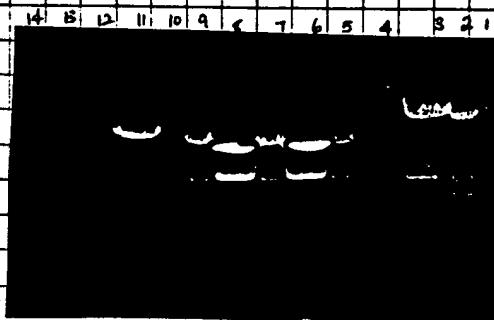
Project No. 407-000000

TITLE

Book No. 000-0000

From Page No. 3/25/91

- 1- MW Marker
- 2- pS134 HindIII
- 3- pST34 HindIII + NcoI
- 4- pS132 NcoI + SacI
- 5- pUC18 link HindIII
- 6- pUC18 link Nco + HindIII
- 7- pUC18 link Nco
- 8- pUC18 link Nco + SacI
- 9- pUC18 SacI
- 10- pUC18 V<sub>129</sub> EcoRV
- 11- pUC18 V<sub>129</sub> EcoRV - HindIII
- 12- pUC18 V<sub>129</sub> PvuII
- 13- pUC18 V<sub>129</sub> PvuII + SacI
- 14- MW Marker



↑ ↑ ↑ ↑  
Isolated these fragments  
and gene cleaned.

Transformed the following DNAs into DH5-α:

S132: V<sub>1308</sub> + signal in 2627 derived vectorS134: V<sub>1308</sub> + signal in 2627 derived vector

S136: 5401-2627 + pCMVPA #1 (from Sid)

Plated on LBamp

\*Result: approximately 300 colonies / plate

Digest pUC18 link w/ EcoRI + HindIII (for frag. isolation + insertion)

Ligations:

	pUC18 82	pUC18 Nco-Sac	pUC18 34	pUC18 Nco-Hind	To Pag
pUC18 Nco-Sac	1ul	1ul	-	-	
S132 Nco-Sac	4ul	-	-	-	
pUC18 Nco-Hind	-	-	1ul	1ul	
S134 Nco-Hind	-	-	4ul	-	
10X Lig. Buf.	2ul	2ul	2ul	2ul	
10X ATP	2ul	2ul	2ul	2ul	
Witnessed & Understood by me,	Date 15 Jul	Investigator by <i>[Signature]</i>	Date 3/25/91		
		Recorded by			

Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_ TITLE \_\_\_\_\_

Form Page No. 3/25/91

PCR Reaction

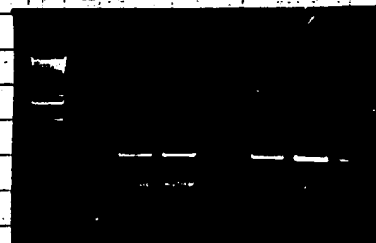
Tube	Fragment	Template	Primers	Program
A	98-6 cDNA HuCKappa W 815' V1129 overlap	98-6 cDNA	SJ114 SJ116	94°C 2min 94°C 11min } 20x 50°C 2min 72°C 2min 8°C soak
B	V1129 W HuCKappa overlap	V1129 1103-104	SJ103 SJ115	

$$\begin{aligned} \text{SJ114: } (330)(40) &= 13,200 \\ (13,200)^{-1} (1 \times 10^{-3}) &= 7.57 \times 10^{-8} = 75.7 \text{ pmol/l} \\ 25(100) &= x(75.7) \\ x &= 33.02 \mu\text{l} \end{aligned}$$

$$\begin{aligned} \text{SJ116: } (330)(30) &= 9900 \\ (9900)^{-1} (1 \times 10^{-3}) &= 100 \text{ pmol/l} \\ 25(100) &= x(100) \\ x &= 25 \mu\text{l} \end{aligned}$$

$$\begin{aligned} \text{SJ103: } (330)(38) &= 12,540 \\ (12,540)^{-1} (1 \times 10^{-3}) &= 7.97 \times 10^{-8} = 79.7 \text{ pmol/l} \\ 25(100) &= x(79.7) \\ x &= 31.37 \mu\text{l} \end{aligned}$$

$$\begin{aligned} \text{SJ115: } (330)(40) &= 13,200 \\ 75.7 \text{ pmol/l} \\ x &= 33.02 \mu\text{l} \end{aligned}$$



To Page No.

Witnessed & Understood by me,

Date

Invested by

Recorded by

Date

3/25/91

From Page No. 40 3/26/91

## Overlapping PCR

Tube	Fragment	Template	Primers	Program
A	mV <sub>1129</sub> -HuCK	mV <sub>1129</sub> 103-105 (1 $\mu$ l)	SJ103 (25 pmol/ $\lambda$ )	94°C 2mi
(1-4)		huCK 114-116 (1 $\mu$ l)	SJ116 (25 pmol/ $\lambda$ )	94°C 1min
				50°C 2mi
				72°C 2mi
				8°C soak
				55°C 2mi
				72°C 2mi
				8°C soak

← isolate these fragments after  
digestion with EcoRI-HindIII

- pooled mV<sub>1129</sub>-HuCK PCR reactions; phenol/chloroform, chloroform, ethanol ppt, &
- digested w/ EcoRI-HindIII (for cloning into pUC18) -
- gel purified and GeneClean

← isolated  
both fragments

Note: Actually there is no HindIII site in this fragment; the 5' and 3' ends should contain EcoRI & sites

To Page 1

Witnessed &amp; Understood by me,

Date

Invented by

Dana J. Blumett

Date

3/26/91

Recorded by

Project No. \_\_\_\_\_

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Page No. 40

2/26/91 huCKappa 114-116 fragments

- pooled mv 1129 103-115 fragments; cleaned, ethanol ppted, resuspended in 40ul TE.
- removed 20ul of ea., brought volume to 35ul
- digested both fragments w EcoRI-HindIII for cloning into pUC18
- gel purified and gene cleaned

Eco-Hind  
mv 1129Eco-Hind  
huCK

## Ligations:

	pUC18 mv 1129 103-115	pUC18 huCK 114-116	pUC18 Eco-Hind
mv 1129 EcoRI-HindIII 103-115	4ul	-	-
huCK EcoRI-HindIII 114-116	-	4ul	-
pUC18(linE) EcoRI-HindIII	1ul	1ul	1ul
TE	10ul	10ul	14ul
Ligase Buf (10x)	2ul	2ul	2ul
60 mM ATP	2ul	2ul	2ul
Ligase	1ul	1ul	1ul
O/N at 4°C			

7. Transformed ligations into DH5- $\alpha$ 

Plated on LB Amp plates after 1 hr. outgrowth in S.O.C.

+X-gal

8

## RESULTS:

# colonies

pUC18 Eco-Hind	~200 white
pUC18/103-115	~1000 colonies, white, blue, light blue
pUC18/114-116	~1000 colonies, white, blue, dark blue

To Page No. \_\_\_\_\_

Reviewed &amp; Understood by me,

Date \_\_\_\_\_

Invented by

Lisa L. Bennett

Recorded by

Date

3/28/91

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

From Page No. 39

3/26/91

Transformed ligated DNAs into DH5- $\alpha$ :

5  $\mu$ l pUC18 Nco-SacT (negative control)

5  $\mu$ l pUC18-SJ32 Nco-Sac

5  $\mu$ l pUC18 Nco-HindIII

5  $\mu$ l pUC18-SJ34 Nco-HindIII

plated on LBamp plates after 1.5 hr outgrowth

Transformed SJ33 and SJ35 DNAs to make stock

(see Sid's notebook for details and description of plasmids)

- into DH5- $\alpha$

- 1  $\mu$ l SJ33 and SJ35

- plated on LBamp after 1.5 hr outgrowth

Transformed SJ36+0(117-118) (Sid's ligation reactions) into DH5- $\alpha$

5  $\mu$ l of SJ36+(117-118) [+]

5  $\mu$ l SJ36 religation [-]

plated on LBamp after 1.5 hr outgrowth

### Results:

pUC18 Nco-Sac	177	uniform
pUC18/32	600-1000	uniform colony size
pUC18 Nco-HindIII	250	uniform
pUC18/34	~500-600	big and small colonies
SJ33	~1000	very small colonies
SJ35	~5000	uniform
SJ36+(117-118)	150	ligations did not appear to work
SJ36 -	150	

To Page

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From Page No. 43

3/28/91

Picked colonies for minipreps and restriction digestion analysis:

Results

S33 1-3

very slow growing

S35 4-6

pUC18/34 (7-10 big) &amp; (11-14 small)

11, 12, 14 very slow growing

pUC18/32 15-22

grew in LB Amp O/N

pS133: hvi308FV Nco + HindIII

pS135: hvi308FCA V-V<sub>H</sub> Nco + BamHI

pUC18/34

pUC18/32

1- pS135 Nco-HindIII #4

2- #5

3- #6

4- pUC18/34 Nco-HindIII #7

5- #8

6- #9

7- #10

8- #13

9- pUC18/32 Nco-SacI #14

10- #15

11- #16

12- #17

13- #18

14- #19

15- #20

16- #21

#22

pick #7 (pUC18/34) } to grow for midi prep  
#15 (pUC18/32) } culture.(Neo-Not)  
4/2 Completed midi prep for pUC18/34 #7  
pUC18(Neo-Not)/82  
pS135

To Page No.

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111

Project No. \_\_\_\_\_

TITLE \_\_\_\_\_

Book No. \_\_\_\_\_

From Page No. 41

Construction of pUC18 - mv<sub>1129</sub> - hucK

Ligation Reactions:

	mv <sub>1129</sub> -hucK	pUC18 EcoRI
mv <sub>1129</sub> -hucK	1µl	1µl
pUC18 EcoRI	4µl	-
TE	10µl	14µl
Ligase Buffer (10x)	2µl	2µl
10mm ATP	2µl	2µl
Ligase	1µl	1µl

incubated at 4°C for 1.5 hr.

- pUC18 (Nco-Not)	10µl
TE	26µl
React 3	4µl
EcoRI (100/µl)	2µl

digested at 37°C 1.5 hr., phenol/chloroform, chloroform, ethanol ppt, wash

transformed ligated DNA into DH5-α

plated on LBamp + X-gal after outgrowth (1 hr.) in SOC

328 Result:

pUC18 mv<sub>1129</sub>-hucK  
pUC18 EcoRI

~ 2000 colonies  
~ 2000 colonies

no blue colonies however  
Explanation: this vector  
is out of frame w/ X-gal  
so unless a gene which  
is in frame is inserted  
all colonies will be white

Note: Better to store

10/2/00

Witnessed &amp; Understood by me:

Date:

Invented by:

David J. Bennett

Date:

Recorded by:

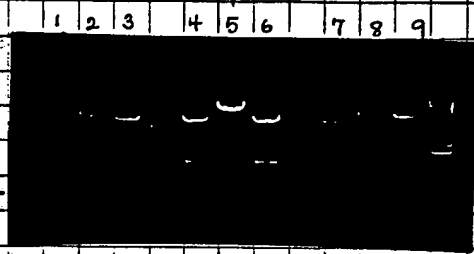
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Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

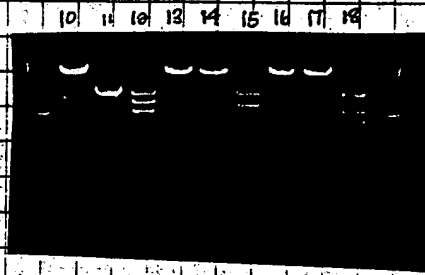
From Page No. 3/27/91

Restriction enzyme digestion analysis of SL32, SL34, SL36, pCMVcat

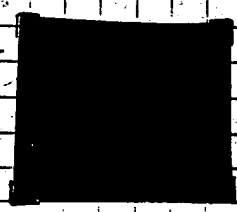
1- SL32 Nco+ Sac }  
2- SL32 Nco }  
3- SL32 Sac }  
4- SL34 Nco-HindIII }  
5- SL34 Nco }  
6- SL34 HindIII }  
7- SL36 EcoRI-BamHI  
8- SL36 HindIII  
9- SL36 SalI  
10- pCMVcat ClaI



11- EcoRV  
12- EcoPI  
13- BglII  
14- SalI  
15- SmaI  
16- HindIII  
17- BamHI  
18- EcoRI-Bam



Preparative Gel to isolate pSL36 HindIII-SalI fragment



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Recorded by \_\_\_\_\_

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To Page No. \_\_\_\_\_



From Page No. 47

3/28/91 SJ36 + (Nco-NotI linker) Construction

- Isolated SJ36 HindIII-SalI fragment from preparative gel
- Gene Created
- Annealed 1  $\mu$ l SJ110 + 1  $\mu$ l SJ111 (1  $\mu$ g/2) in 1x Reaction Buf. <sup>14  $\mu$ l</sup> ~~2  $\mu$ l~~ H<sub>2</sub>O  
2 min 65°C, cooled slowly to room temp.
- Ligated SJ36 HindIII-SalI + (SJ110-SJ111)

		control
PSJ36 HindIII-SalI f.	2 $\mu$ l	2 $\mu$ l
SJ110-SJ111	2 $\mu$ l	-
H <sub>2</sub> O	11 $\mu$ l	13 $\mu$ l
10x Ligase Buffer	2 $\mu$ l	2 $\mu$ l
10mM ATP	2 $\mu$ l	2 $\mu$ l
Ligase	1 $\mu$ l	1 $\mu$ l
incubated at room temperature for 3 hr.		

- Transformed into DH5- $\alpha$  and plated on LB Amp media
- Incubate at RT over weekend

- Transformation did not work: probably because one of the enzymes did not cut all the way.

The DNA was cut w/ HindIII + SalI in React 3  
HindIII doesn't cut well in React 2.

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X T Bennett

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3/28/91

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Project No. \_\_\_\_\_

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From Page No. 42

• Picked colonies to grow minipreps and analyze by restriction digestion

4/1

1-5) pUC18/V. 1129 103-115 blue

6-10) pUC18/V. 1129 103-115 white

11-15) pUC18/huCK 114-116 blue

16-20) pUC18/huCK 114-116 white

21-36) pUC18/mv. 1129 + huCK (white)

• Kept in refrigerator

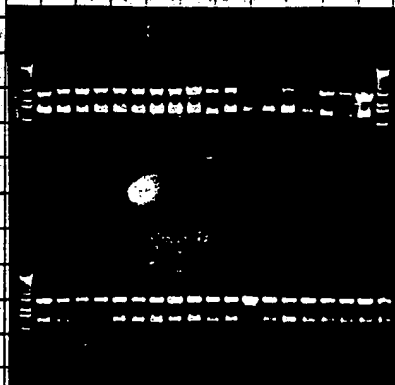
4/2

Completed minipreps and analyzed by restriction digestion

1-10) pUC18(Nco-Not)/V. 1129 103-115 EcoRI + HindIII

11-20) pUC18(Nco-Not)/huCK 114-116 EcoRI + HindIII

21-36) pUC18(Nco-Not)/mv. 1129 + huCK EcoRI



To Page N

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*Al Bennett*

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From Page No. \_\_\_\_\_

4/1/91

INSERTION OF NcoI-NotI linker INTO pS136

Digest<sup>ed</sup> pS136 with HindIII + Salt

36µl pS136

4µl Restr 2

2µl HindIII

incubated 1hr at 37°C

added 0.6µl 3M NaCl

2µl SalI

incubated 1hr at 37°C

heat killed

fractionated prep on 1% agarose gel

GeneCleaned

1- pS136 HindIII-Sal

2- pUC18(Nco-Not) EcoRI-BamHI

3- pUC18 V<sub>L</sub>-V<sub>H</sub> 1127 EcoRI-BamHI



Ligation

pS136 HindIII-SalI

S1110 + S1111

10X Ligase Buffer

10mM ATP

H<sub>2</sub>O

Ligase

incubated at 4°C O/N

pS136 Hind-SalI  
S1110-III

1µl

3µl

2µl

2µl

1µl

1µl

pS136 Hind-SalI

1µl

-

2µl

2µl

14µl

1µl

4/2/91

Transformed into DH5- $\alpha$  and plated on LB Amp

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 Invented by L. Bennett Date 4/2/91  
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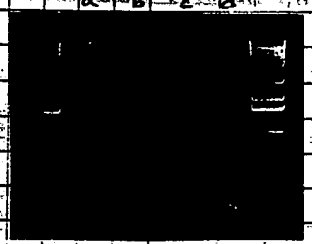
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4/1/91

overlapping PCR Reaction to generate mV<sub>1129</sub> - hUCKappa chimera

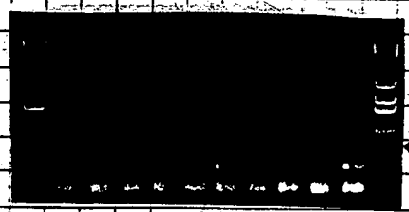
Template	Fragment	Primers	Program
mV <sub>1129</sub> (b3-b5)	mV <sub>1129</sub> -hUCK	5' 103 (100pmol/2)	94°C 1 min } 65°C 2 min } 30X 72°C 2 min } 8°C soak
hUCK(114-116)		3' 116 (100pmol/2)	
(purified frag.)			
a b c d			



• Threw these reaction out because previous experiment (at 50°C) was better

4/2/91

• Diluted template DNA 1/10 and 1/100 and reran the exp at 50°C annealing temp



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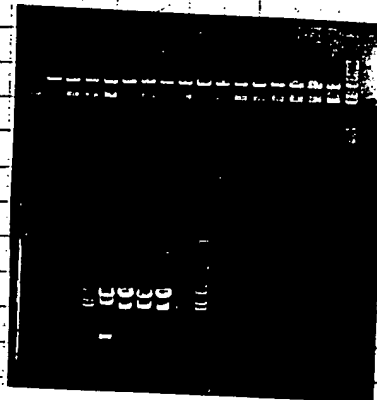
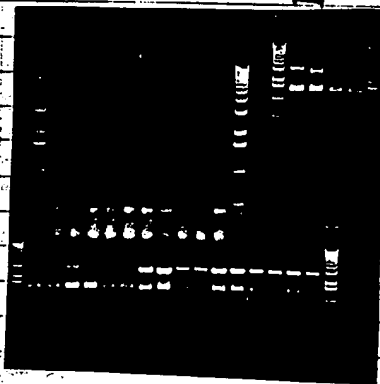
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From Page No. 44

4/3/91

Restriction enzyme analysis of pUCN-N huCK and pUCN-N VL-huCK

1- pUCN-N huCK EcoRI #11	21- pUCN-N VL-huCK EcoRI-HindIII #21
2- EcoRI-HindIII #11	#22
3- #12	#23
4- #12	#24
5- #13	#25
6- #13	#26
7- #14	#27
8- #14	#28
9- #15	#29
10- #15	#30
11- #16	#31
12- #16	#32
13- #17	#33
14- #17	#34
15- #18	#35
16- #18	#36
17- #19	37- pSJ35 Neo-HindIII ✓
18- #19	38- pUCN-N Neo-HindIII ✓
19- #20	39- pUCN-N/34 Neo-HindIII ✓
20- #20	40- pUCN-N Neo-Sac ✓
	41- pUCN-N/32 Neo-Sac ✓



none of the puc  
VL-huCKappa ap  
to be correct.

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4/3/91

To Page No.

4

Project No. \_\_\_\_\_

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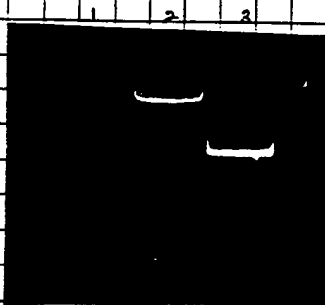
TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

4/3/91

Preparative Gel

- 1- pUCN-N/32 Pvu II-Eco Nco I
- 2- pUCN-N/34 EcoRV-Not
- 3- V<sub>129</sub>-huckappa EcoRI-Not I
- 4- pUCN-N/82 Pvu II-Nco I
- 5- pUCN-N EcoRI-Not I



1- pUCN-N/32 Pvu II

2- Nco I

3- Pvu II-Nco I

4- pUCN-N Pvu II

5- pUCN-N Nco I

6- pUCN-N/34 EcoRV

7- Not I

8- pUCN-N EcoRV

9- pUCN-N Not I

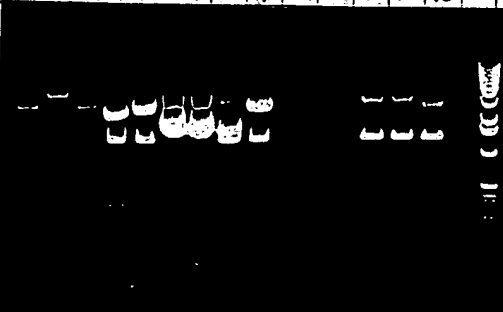
10- V<sub>129</sub>-huckappa f<sub>2</sub> Eco-Not11- pUCN-N V<sub>129</sub> EcoRV

12- Hind III

13- EcoRV-Hind III

overloaded

1 2 3 4 5 6 7 8 9 10 11 12 13



To Page No. \_\_\_\_\_

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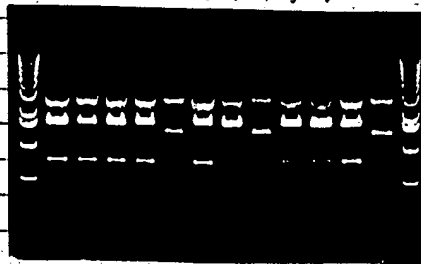
From Page No. \_\_\_\_\_

9/3/91

Miniprep and restriction enzyme analysis of pUC-N-N V<sub>L</sub>-V<sub>H</sub> 1129 & pSJ36 (110-111)

1-12) pUC-N-N V<sub>L</sub>-V<sub>H</sub>

13-24) pSJ36 (110-111)



1-12) pUC-N-N V<sub>L</sub>-V<sub>H</sub> 1129 EcoRI-BamHI

13-24) pSJ36 (110-111) NotI

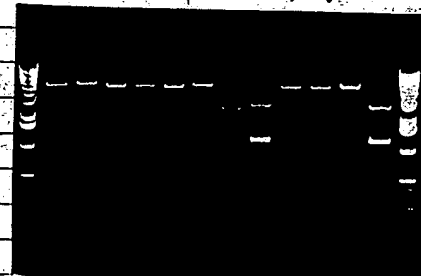
25- pUC-N-N V<sub>L</sub>-V<sub>H</sub> EcoRI (mid)

26- Hind EcoRI

27- Hind

28- Hind+NotI

29- 100bp ladder



Grew midi cultures of #2, #3, 14, 15



To Page \_\_\_\_\_

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4/3/91

Project N 11-11-19Book No. 11-11-19 TITLE \_\_\_\_\_

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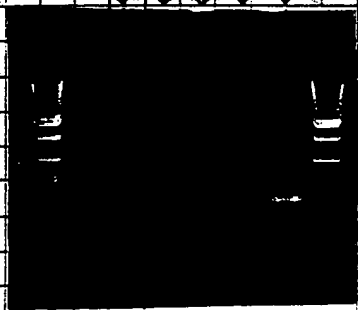
4/4/91

PCR Reactions

Tube	Fragment	Template	Primers	Program
A	V <sub>L</sub> 1129 103-115	<del>pos 1129</del> V <sub>L</sub> 1129 103-104	SI 103 SI 115	94°C 2 min 94°C 1 min 52°C 2 min } 30x 72°C 2 min
B	hucKappa 114-116	p 98-104 hucKappa 114-116	SI 114 SI 116	8°C 30x
C	V <sub>L</sub> 1129-hucKappa	V <sub>L</sub> 1129 103-115 hucKappa 114-116	SI 103 SI 116	
D	hucKappa 114-116	268 SO 128 mp	SI 122 <del>SI 123</del> SI 123	
E	V <sub>L</sub> 1129 103-115	V <sub>L</sub> 1129 103-115	SI 103 SI 115	
F	hucKappa 114-116	hucKappa 114-116	SI 114 SI 116	

4/5/91

Put reaction back in Perkin-Elmer machine for 30 min



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4/4/91



TITLE

Project No. 44740109

Book No. 04 4009

From Page No.

4/5/91

Construction of signal-V<sub>1</sub> 1129-huCKappa in pUC11N

A. Pooled pUC11N/V<sub>1</sub> 1129-103 mini DNAs #1,2,3

Brought volume to 36ul

Added 4ul React 2

2ul EcoRV

2ul HindIII

B. Digested 36ul pUC11N/huCKappa with HindIII + NotI

4ul React 2

2ul HindIII

2ul HindIII NotI

C. Pooled pUC11N/34 min DNAs #7,8,9

Brought volume to 36ul

Added 4ul React 2

2ul EcoRV

2ul NotI

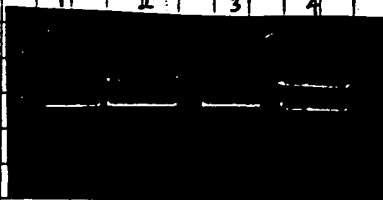
Preparative gel

1- pUC11N-huCKappa HindIII-NotI

2- pUC11N/34 EcoRV-NotI

3- pUC11N V<sub>1</sub> 103-HIS EcoRV-HindIII

4- pUC11N-Nco-SacI



To Page N

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4/5/91

Project No. \_\_\_\_\_

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15/91

ligations

	PUCN-N Signal-V <sub>L</sub> -hucK	PUCN-N Signal-V <sub>H</sub>
PUCN-N/V <sub>H</sub> 129 EcoRV-HindIII	5 $\mu$	-
PUCN-N/hucKappa HindIII-NotI	5 $\mu$	-
PUCN-N/34 EcoRV-NotI	5 $\mu$	-
PUCN-N/32 PvuII-NotI	-	5 $\mu$
PUCN-N Nco-SacI	-	2 $\mu$
V <sub>H</sub> 129 PvuII-SacI p.c.f.	-	5 $\mu$
10x Ligase Buffer	2 $\mu$	2 $\mu$
10mm ATP	2 $\mu$	2 $\mu$
Ligase	1 $\mu$	1 $\mu$
H <sub>2</sub> O	-	3 $\mu$

Incubate at 4°C over weekend

4-8-91

Transformed ligations into DH5-K on 18 Amp-Xgal plates

Also transformed PUCN-N/34 #10, PUCN-N/32 midi, PUCN-N V<sub>H</sub>129 103-115 mini #3

# colonies/plate

PUCN-N/Signal-V<sub>H</sub>129

all white

PUCN-N/Signal-V<sub>L</sub>-hucKappa

&gt;400 with light blue + white

1/9/91

picked 20 PUCN-N/Signal-V<sub>H</sub>129

1-20

20 PUCN-N/Signal-V<sub>L</sub>-hucKappa

21-40

1-20) Nco-PvuII

21-40) EcoRV-HindIII

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Recorded by

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4/9/91

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Job No. \_\_\_\_\_

From Page No. \_\_\_\_\_

4/5/91

Construction of chimeric signal-mV<sub>H</sub>129-huCK in pUCN-N

Digested 100µl pUCN-N/32 with PvuII + NcoI

10µl React 2

4µl PvuII

4µl NcoI

Digested 36µl pUCN-N with NcoI-SacI

4µl React 1

2µl NcoI

2µl SacI

Digested 10µl V<sub>H</sub>129 105-106 PCR fragment w PvuII + SacI

1.3µl React 4

1µl PvuII

1µl SacI

Preparative Gel to isolate pUCN-N/32 60bp PvuII-NcoI fragment

4/8/91

Digested <sup>10µl</sup> huCK PCR fragment with SacI + NotI

1.3µl React 1

1.0µl SacI

1.0µl NotI

incubated 1.5 hr at 37°C

To Page 1

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*STEAMT*

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4/8/91

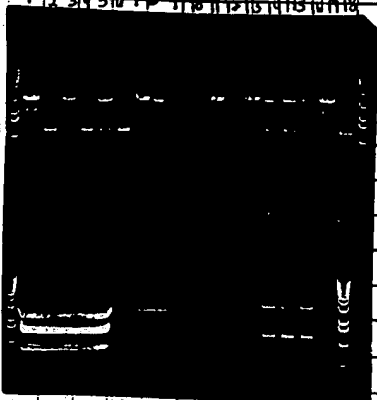
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TITLE \_\_\_\_\_

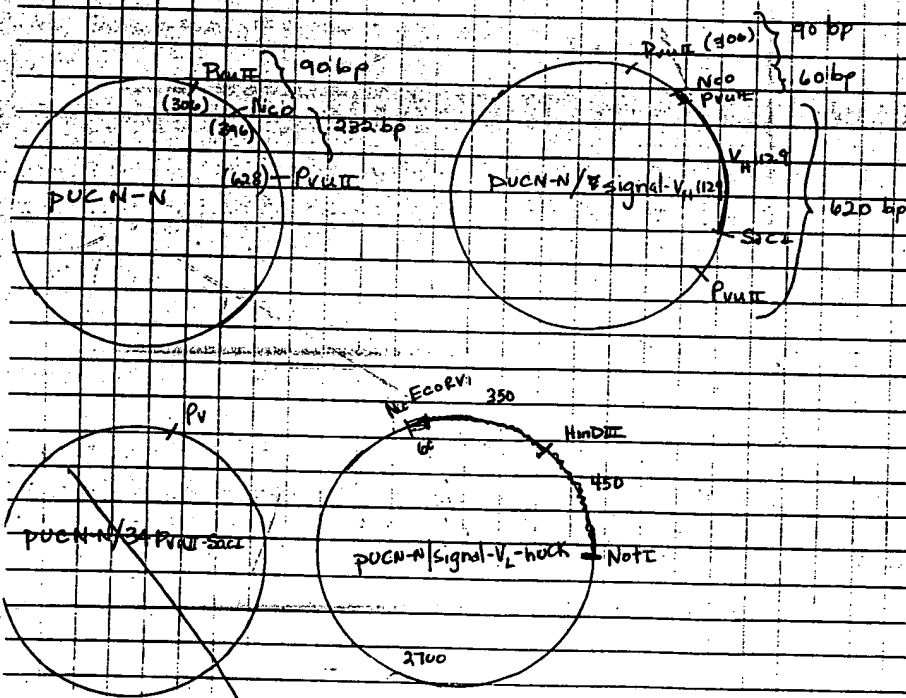
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18



- 1-20) PUCN-N/Sigmal-V<sub>L</sub>-hucKappa-EcoRV-NotI
- 21) PUCN-N/34 EcoRV-NotI

- A) PUCN-N/V<sub>L</sub>-V<sub>H</sub> #3 EcoRV-NotI
- B) PUCN-N/V<sub>L</sub>-V<sub>H</sub> #2 EcoRV-NotI



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Project No. \_\_\_\_\_

TITLE \_\_\_\_\_

Book No. \_\_\_\_\_

From Page No. \_\_\_\_\_

4/5/91

Construction of pUC-N/V<sub>h</sub>-hucKappa by overlapping PCR

Ligation

	pUC-N-N V <sub>h</sub> -hucK	pUC-N-N Control
pUC-N-N EcoRI-NotI	2 $\mu$ l	2 $\mu$ l
V <sub>h</sub> 129-hucKappa EcoRI-NotI	5 $\mu$ l	-
H <sub>2</sub> O	8 $\mu$ l	13 $\mu$ l
Ligase Buffer (10x)	2 $\mu$ l	2 $\mu$ l
10mM ATP	2 $\mu$ l	2 $\mu$ l
Ligase	1 $\mu$ l	1 $\mu$ l

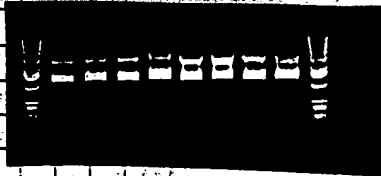
incubated in refmg (4°C) over weekend

4-8-91

Transformed ligations into DHE-a

	# colonies/plate
pUC-N-N control	150
pUC-N-N/V <sub>h</sub> -hucKappa	300 dark medium blue + white

picked 48 blue colonies for mini preps 41-48



46 of 48 positives EcoRI-NotI digestion

#46 grown for midi prep.

To Page 1

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Date \_\_\_\_\_

Invented by

S. Bennett

Recorded by \_\_\_\_\_

Date

4/8/91

Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_ TITLE \_\_\_\_\_

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10/91

Completed midi preps of pUCNN/V<sub>1</sub>-V<sub>4</sub> #2 #3 and pSV36(110-111) #14 #15

1- pUCNN/V<sub>1</sub>-V<sub>4</sub> #2 Sac

2- EcoRV

3- EcoRI-Bam

4- HindIII

5- HindIII-EcoRI

6- pUCNN/V<sub>1</sub>-V<sub>4</sub> #3 Sac

7- EcoRV-Sac

8- EcoRV

9- EcoRI-BamHI

10- HindIII

11- HindIII-EcoRI

12- pSV36(110-111) #14 NeoI

13- NotI

14- SalI

15- HindIII

16- EcoRI-BamHI

17- #15 NeoI

18- NotI

19- SalI

20- HindIII

21- EcoRI-BamHI



5 µl of DNA loaded

?

looks good

EcoRV-Sac digestion of pUCNN/V<sub>1</sub>-V<sub>4</sub> should generate an 0.8 Kb fragment but appears to be only linearizing the plasmid. - Continue restriction digestion analysis to determine problem.

To Page No. \_\_\_\_\_

Inspected & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

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TITLE \_\_\_\_\_

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Book No. \_\_\_\_\_

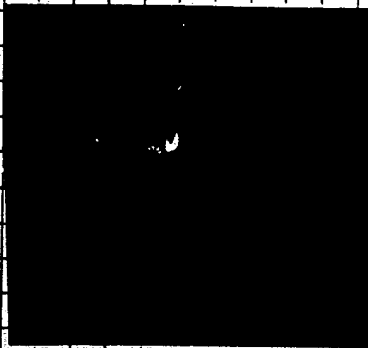
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4/9/91

Preparative gel to isolate hucX, SacI-NotI PCR fragment

pUCN-U/V-V<sub>h</sub> EcoRV-SacI f

V<sub>h</sub>-hucX 103-116 EcoRI-NotI fragment



← hucX, SacI-NotI

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4/9/91

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4-9-91

1- PSJ37 Not

2- DS137 NCO

3-  $\text{pUC1-NV-N}_1$  #3 EcoRV (React 4)

4-					#3 Ecov-Sac)
----	--	--	--	--	--------------

5-						ERFV-NUTZ
----	--	--	--	--	--	-----------

6-						FCORV-HNDM
----	--	--	--	--	--	------------

7-						Cl <sub>2</sub>
----	--	--	--	--	--	-----------------

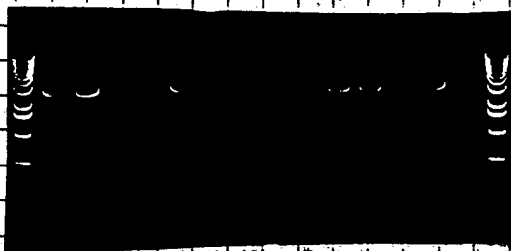
8.							Ch-Sect
----	--	--	--	--	--	--	---------

-9-								Ca-Not
-----	--	--	--	--	--	--	--	--------

[illegible]

11-					EcoRV (React 2
-----	--	--	--	--	----------------

did not aut



Digested 36 pl. PUCN-NV-V #3

4. React 2

2nd Net I

2.0 Ecov

incubated at 37°C

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4/9/91

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4-11-91

Ligations

PUCN-N/34  
control

PUZ N + N / 34  
U<sub>1</sub> - V<sub>4</sub> # 3

SUN-N/34  
V-V #2

DUCN-N/84 EcoRV-Not

2

1

V-V<sub>u</sub> #2 EcoRV-Not f

1

†

五

$v_1 - v_4 \neq 3$  EcoRV-Not f.

—

5

1

Lia Buffer

2

2

6

H<sub>2</sub>O

4

1

1

**Ligase**

1

**T**

1	2
---	---

0	10mm	ATP
---	------	-----

2

[illegible]

--	--

refrigerated (4°C) until return

4/23/91

Transformed DH5- $\alpha$  comp. cells with the above ligation mixtures

- 1 hr. outgrowth

- plated on LB Amino

Transformed into  $\lambda$ H5- $\alpha$  and plated on LB Amp + X-gal.

Page N

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## Inventories

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4/11/9

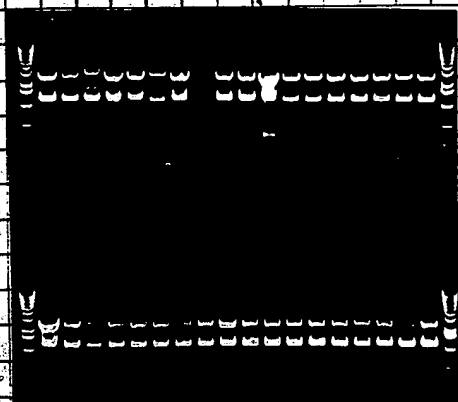
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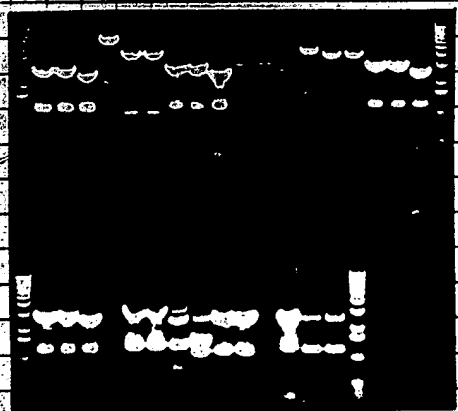
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- 1-10) pUCN-N/V<sub>L</sub>-huCKappa EcoRI-HindIII #1-10
- 11) pUCN-N V<sub>L</sub> 103-104 EcoRI + HindIII
- 12-18) pUCN-N/ S-V<sub>L</sub>-huCKappa EcoRI + HindIII # 11-1
- 19) pUCN-N/V<sub>L</sub> 103-104 EcoRI + HindIII
- 20-34) pUCN-N/S-V<sub>L</sub>-huCKappa #18-34 EcoRI + H



- 1- pUCN-N/34 Nco
- 2- HindIII
- 3- Nco + HindIII
- 4- SJ34 Nco
- 5- HindIII
- 6- Nco + HindIII
- 7- pUCN-N/34 (new) Nco
- 8- HindIII
- 9- Nco + HindIII
- 10- pUCN-N/32 Nco
- 11- Sac
- 12- Nco + Sac
- 13- SJ32 Nco
- 14- Sac
- 15- Nco + Sac
- 16- pUCN-N/32 Nco
- 17- Sac
- 18- Nco + Sac
- 19- pUCN-N/V<sub>L</sub> 103-104 Hind
- 20- EcoRI
- 21- HindIII
- 22- pUCN-N V<sub>L</sub>-V<sub>H</sub> 103-104 Ec
- 23- N
- 24- Eco
- 25- pUCN-N EcoRV
- 26- Not
- 27- EcoRV + Not
- 28- pUCN-N V<sub>L</sub> 103-104 EcoRI
- 29- pUCN-N/S-V<sub>L</sub>-huCKappa #35
- 30- " #36
- 31

To Page

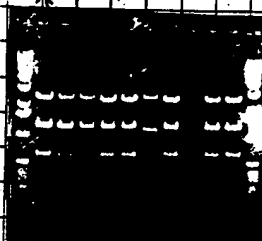
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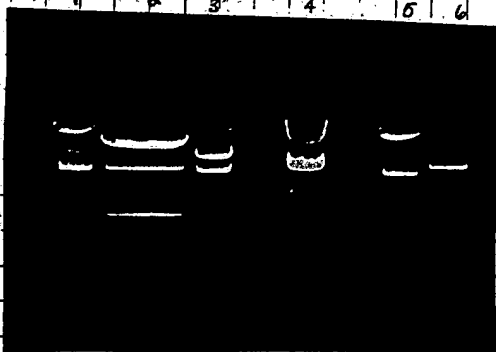
Page No. 4/25/91

\* Sequenced



1-10) pUCN-N/V-huCKappa EcoRT + NotI

\* all but 1 and 8 are potentially correct  
 however, #3 is slightly bigger  
 should sequence several to determine if actually correct



1- pUCN-N/34 EcoRV-NotI

2- pUCN-N/32 Nco-PvuII

3- pUCN-N/34 EcoRV-Sac

4- pUCN-N/V-V EcoRV-Sac

5- pUCN-N Nco-Sac

6- pUCN-N/mv-huCK (Alt 48) EcoRV-NotI

Gene Cleared - pUCN-N/34 EcoRV-Not

pUCN-N/34 EcoRV-Sac

pUCN-N Nco-Sac

Freeze-squeeze

pUCN-N/32 Nco-Pvu II

pUCN-N/V-V EcoRV-Sac f

pUCN-N/mv-huCK EcoRV-Not f

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From Page No. ~~4/16/91~~

4/30/91

Sequencing reactions  
Template

Primer

1-	pUCN-N/V <sub>L</sub> -hucKappa	#46 midi	reverse (S112) UP
2-		#48 midi	"
3-		#1 mini	"
4-		#4 mini	"
5-		#48	S1112
6-		#44	"
7-		#1	"
8-		#4	"
9-	//	#46	reverse (S112)
10-		#48	
11-		#4	

Primer

1-	pUCN-N/S-V <sub>H</sub>	#3 mini DNA	UP
2-		#4	"
3-		#5	"
4-		#7	"
5-		#8	"
6-		#9	"
7-		#10	"
8-		#11	"
9-		#16	"
10		#18	"

Notes: sequencing reactions on mini DNAs did not work  
 ∴ probably not enough DNA

Grew midi cultures (50 ml) of pUCN-N/V<sub>L</sub>-hucK #1, #4  
 pUCN-N/S-V<sub>H</sub> #4, 5, 9, 18

#46 & #48 were pUCN-N/V<sub>L</sub>-hucKappa which ~~was~~ blue; sequencing showed 46 col  
 a two-base-pair deletion in linker region & 48 has a one base pair Δ. To Page

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5/1/91

Digested 36ul of pS41 (pUCN-1/34) with ~~Hand~~ EcoRV and NotI  
 4ul React B  
 2ul EcoRV  
 2ul NotI  
 Incubated for 1hr at 37°C

Fractionated on 1% agarose gel O/N

To Page

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From Page No. 5/6/9

8 Developed the following sequencing gel

1- } #4

2- } #5

3- } #9

4- } #18

S-V<sub>H</sub> 1129 potential clones

up primer ?

5- } #1 UP

6- } #1 S112

7- } #1 reverse

mv<sub>L</sub> - huCKappa

← sequence is correct

8- } #4 UP

9- } #4 S112

10- } #4 reverse

mv<sub>L</sub> 1129 - huCKappa

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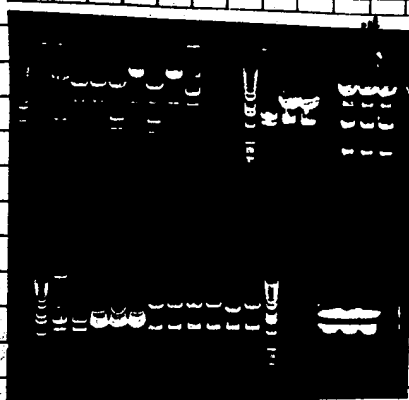
To Page N

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

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From Page No. 5/7/91



- |                            |                       |
|----------------------------|-----------------------|
| 1- <del>pS134</del> MW Std | 15- pS141 uncut       |
| 2- pS134 uncut             | 16- EcoRV             |
| 3- EcoRV                   | 17- EcoRV             |
| 4- RV-HindIII              | 18- EcoRV-Sac         |
| 5- RV-HindIII              | 19- Sac               |
| 6- HindIII                 | 20- EcoRV-Hind        |
| 7- NcoI                    | 21- Hind              |
| 8- EcoRV-SacI              | 22- RV-Not            |
| 9- SacI                    | 23- Nco Not           |
| 10- NotI                   | 24- Nco-Not           |
| 11- pS140 uncut            | 25- Nco               |
| 12- HindIII                | 26- pS140 HindIII-Nco |
| 13- Nco                    |                       |
| 14- pS134 Nco+HindIII      |                       |

Note: pS141 does not appear to have an EcoRI site.  
 pS134 however has two ~~for~~ NcoI-HindIII fragments of about 400bp  
 therefore pS141 probably contains the wrong Nco-HindIII frag  
 Will redo the construction and screen minipreps with EcoRV+Hind

Isolated pS134 Nco+HindIII 400bp fragments  
 pS140 Nco+HindIII 2.5 Kb fragment

Ligations	pS140 Nco-Hind	pS140 Nco-HindIII
pS140 Nco+HindIII 2.7 Kb f	1ul	1ul
pS134 Nco+HindIII 400bp fragments	6ul	-
10x Ligase Buffer	2ul	2ul
10mm ATP	2ul	2ul
H <sub>2</sub> O	4ul	14ul
Ligase	1ul	1ul

incubated 12 hr. at room temperature.

transformed into comp. DH5- $\alpha$  (Sid's cells)

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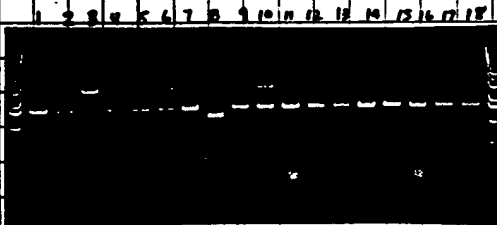
Project No. \_\_\_\_\_

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From Page No. \_\_\_\_\_

Construction of  $mV_H$  -  $h_{21}$  mixture.



1-18) potential p5140 S-V, 1129. Nao - Pruit

No clones appear to be correct

Result: This three-part ligation strategy is not working. Need a new plan.

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Project No.                     Book No.                      TITLE                     om Page No.                     Sequence (Triplets): CCG AAT TCG CAC TCA TTT ACCCGG AGA CAG G

SJ21

1.39 mg/ml

human C8 3' primer

Sequence (Triplets): GTC ACC GTG AGC TCA GCC TCCACC AAG G

Sa-I

C8 5' primer

SJ22

1.99 mg/ml

Sequence (Triplets): CGG AAT TCA GGT TTA TCT GCAGTA GTC WGG

R+I

SJ42

2.44 mg/ml

Sequence (Triplets): CGG AAT TCA GGT SMA RCT GCAGSA GTC WGG

[E][A][A]

[G]

[A]

SJ43

1.72 mg/ml

Sequence (Triplets): CGG AAT TCA GGT ~~CAA~~ GGT GCAGCA GTC TGG

GCA

SJ44

2.92 mg/ml

Sequence (Triplets):                     

10

20

GCGAATTC ATGGACTGGA OCTGGAGGGT C

5' section AB of humanized 1308F V-H

SJ34

2.033 mg/ml

Sequence (Triplets): GGC GGA TCC GAG GTG CAG CAGCTG GTG CAG

257 VH '5 (PvuII)

SJ71

3.96 mg/ml

To Page N                     Recorded by                     

5/3/91

TITLE

Project No.

Book No.

From Page No. 5-8-91

Isolation of 447 (HVI Mab) from mouse-human hybrid cell line.

A. Isolated poly A<sup>+</sup> RNA (mRNA) using in vitro Nicro Fast Track kit  
- see instructions w/ kit.

B. Generated cDNA from 1/3 volume poly A<sup>+</sup> 447 mRNA Jul  
- see kit instructions

C. Ethanol ppt washed and resuspended in 20ul volume H<sub>2</sub>O  
phenol extracted

D. PCR Reaction using the following primer combinations:

Tube	Template	Primer
A	447 cDNA	S121 S173
B	"	S121 S172
C	"	S121 S143
D	"	S121 S144
E	"	S121 S184
F	"	S121 S182
G	"	S121 S122
H	"	S123 S122

Template	DNA	Volume
10x PCR Buf		10ul
bx dNTP		10ul
Primer #1		1ul
Primer #2		1ul
Taq Pol		1ul
H <sub>2</sub> O		67.5ul

Also wanted to do rxn  
with S121 + S171 but  
could not find S171.

To Page No.

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Lisa L. Bennett

Date

5/8/91

Invented by

Recorded by

Date

6/2/91

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From Page No. \_\_\_\_\_

Sequence (Triplets): \_\_\_\_\_ CAG GTG CAG CTG  
 CAG GTG TCG GGC CC  
 V<sub>H</sub> 248 (5'-Pst PvuII)  
 SJ73 2.49 mg/ml

Sequence (Triplets): GGG AAT CGA GCT CGT CAT GAA  
 ACA CCT GTG G  
 Ⓢ SJ82 4.64 mg/ml  
 BspHI at natural V<sub>H</sub> signal of 98-6

SJ119 Hu1308VH/H71 arg to ser (top)  
 5' AgA gTC ACC ATg ACC TCA gAC ACg TCC ACg AgC

SJ120 Hu1308VH/H71 arg to ser (bottom)  
 5' gCT CgT ggA CgT gTC TgA ggT CAT ggT gAC TCT

SJ121 Hu1308VH/mu to hu 66-71 (top)  
 5' AAg gCC AgT ATT ACA TCA gAC ACg TCC ACg AgC

SJ122 Hu1308VH/mu to hu 66-71 (bottom)  
 5' gAT gTA ATA CTg gCC TTg CCC Tgg AAC TTC ggg

SJ123 Human C-gamma 3' / RI, NotI  
 5' CCg AAT TCg Cgg CCg CAC TCA TTT ACC Cgg AgA CAg g

Witnessed & Understood by me, Lisa Bennett Date 5/8/97 Invented by \_\_\_\_\_ Date \_\_\_\_\_  
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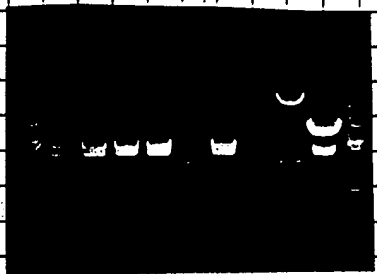
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From Page No. 5-9-91

Construction of pSJ40 containing pSJ34 Neo-HindIII fragment



- 1- uncut 40-34 #3
- 2- 40-34 #3 EcoRV
- 3- #4 EcoRV
- 4- #5 EcoRV
- 5- #6 EcoRV ✓
- 6- #7 EcoRV
- 7- #9 EcoRV ✓
- 8- pSJ34 EcoRV
- 9- pSJ40 HindIII

Using LBamp + 5% X-gal, showed that clone #7 turns blue while potential positive clones #6 and #9 remain white. This result is the opposite of what was expected. The HindIII and the SacI sites are supposedly in frame with lacZ.

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Lisa J. Borel

Date

5/2/91

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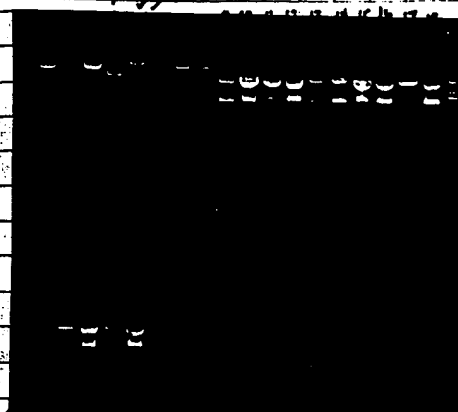
Date

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- |                      |                         |
|----------------------|-------------------------|
| 1- pS132 Nco-Sac     | 12- pS142 Nco-Sac       |
| 2- "                 | 13- pS144 HindIII       |
| 3- pS134 RV-Hind     | 14- pS144 HindIII       |
| 4- "                 | 15- pS145 EcoRI-HindIII |
| 5- pS136 BamHI-EcoRI | 16- "                   |
| 6- "                 | 17- pS146 SalI-BamHI    |
| 7- pS137 Nco-EcoRI   | 18- "                   |
| 8- "                 | 19- pS147 EcoRI-Bam     |
| 9- pS140 Nco         | 20- "                   |
| 10- "                | 21- pS148 EcoRI-Bam     |
| 11- pS142 Nco-Sac    | 22- "                   |

To Page

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*Lisa Bennett*

Date

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Date

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From Page No. 5-14-91	
1-	PS141-6 EcoRI
2-	EcoRI-HindIII
3-	HindIII
4-	Nco-HindIII
5-	Nco
6-	NotI
7-	EcoRV-NotI
8-	EcoRV
9-	PS141-9 EcoRI
10-	HindIII
11-	Nco-HindIII
12-	Nco
13-	NotI
14-	RV-NotI
15-	EcoRV
16-	PS149 EcoRV
17-	EcoRV-NotI
18-	NotI
19-	PS143 EcoRV
20-	EcoRV-Not
21-	Not
Preparative gels	
1-	PS141-6 EcoRV-Not no DNA
2-	PS141-9 EcoRV-Not very dilute
3-	PS143 EcoRV-Not isolated
4-	V#1129 PvuII-SacI
43 V#1129	

Witnessed & Understood by me,

*Kasi Bennett*

Date

5/11/97

Invented by

Recorded by

Date

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m Page No. 5-15-91

Ligations	41 control	41 43	41 49	VH- pST32(0.4)	VH- pST32(0.8)
pST41 EcoRV-NotI f.	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l	-	-
pST43 EcoRV-NotI f.	-	5 $\mu$ l	-	-	-
pST49 EcoRV-NotI f.	-	-	5 $\mu$ l	-	-
pST32 BglI-PvuII, BglI-SacI	-	-	-	2 $\mu$ l	2 $\mu$ l
VH 1129 0.4 B-PvuII-SacI	-	-	-	5 $\mu$ l	-
VH 1129 0.8 PvuII-SacI	-	-	-	-	5 $\mu$ l
Buffer	4 $\mu$ l	4 $\mu$ l	4 $\mu$ l	4 $\mu$ l	4 $\mu$ l
Ligase	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l
H <sub>2</sub> O	13 $\mu$ l	8 $\mu$ l	8 $\mu$ l	8 $\mu$ l	8 $\mu$ l

incubated at RT for 3 hr.

transformed into DH5- $\alpha$ , plated on LB Amp

5-16-91

Pick	6	pST32 VH (0.4)	} grew 5ml minipreps
	6	pST32 VH (0.8)	
	<del>41-43</del>		
	12	41-43	
	12	41-49	

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5/15/91

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From Page No. 86 5-17-91

## Digestion of minipreps

1- S-V<sub>H</sub> (0.4) #1-6 Nco-Sac2- S-V<sub>H</sub> (0.8) #7-12 Nco-Sac

3- 41-43 #14

4- #16

5- #18

6- #20

7- #21

8- 41-49 #25

9- #26

10- #29

11- #31

12- #33

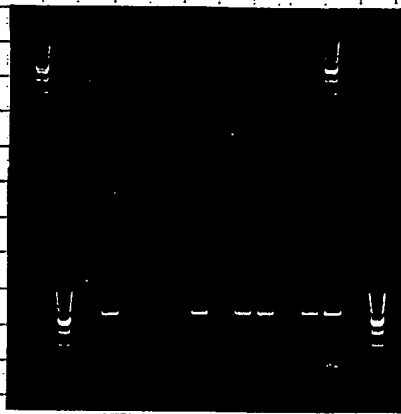
13- #34

14- #35

15- #36

Nco-SacI

Nco-NotI

1- S-V<sub>H</sub> #1 BglI-Xho

2- #2

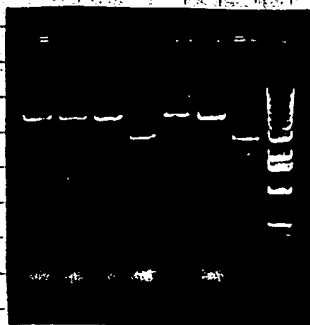
3- #3

4- #4

5- #5

6- #6

7- pST32



To Page

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5/17/91



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21/91

Transformed miniprep samples #1, #2, #6, #18, #21, #26, #33 into HB101 comp. cells  
Plated on LB Amp.

22/91

1- S-V<sub>H</sub> #1 Neo-Pr<sup>II</sup>

2- " #2 "

3- S-V<sub>H</sub> #6 "4- S-V<sub>H</sub> #1 BglI-XhoI5- S-V<sub>H</sub> #2 BglI-XhoI6- S-V<sub>H</sub> #6 BglI-XhoI

7- 41-43 #18 Neo-SacI

8- 41-43 #21 Neo-SacI

9- 41-49 #26 Neo-Not

10- 41-49 #33 Neo-Not

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Recorded by \_\_\_\_\_

Date 5/21/92

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TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

From Page No. 5/21/91

PCR Reaction to amplify huC<sub>8</sub>

Template	Fragment	Primers	Program
268 SD.125 mp	huC <sub>8</sub>	ST122 ST123	94°C 2min 94°C 1min 52°C 2min 72°C 2min } 30

phenol: chloroform, chloroform, ethanol ppt, ethanol wash  
 digest with SacI and EcoRI in React 4  
 digest pUC18 with SacI and EcoRI

5/22/91

preparative gel of huC<sub>8</sub>, EcoRI-Sac  
 isolated and Gene Cleaned

pUC18 EcoRI-SacI  
 isolated and Gene Clean

To Page

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5/24/91

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TITLE \_\_\_\_\_

m Page No. 5/23

ligations

puc18

puc18/hucl

puc18 EcoRI - Sac

2  $\mu$ l2  $\mu$ l

hucl, EcoRI - Sac PCR f.

-

5  $\mu$ lH<sub>2</sub>O13  $\mu$ l8  $\mu$ l

Buffer

4  $\mu$ l4  $\mu$ l

Ligase

1  $\mu$ l1  $\mu$ l

incubated at RT for 2 hr.

transformed into DH5- $\alpha$  and plated on LBamp.

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Western blot

	amount
1- MW std.	
2- V. EH CS #4 (neg. control)	10ul -
3- dhr FR positive control	10ul -
4- RSV long sup	15ul
5- RSV Hep2 sup	15ul
6- HEP2 sup	15ul
7- HEP2 pellet	15ul



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Date 5/23/91

Exhibit 3

David S. Pfarr Notebook References for Humanized IgG Expression

Expression Notebook	Vector Construction Procedure	Pages	Date
87	MIMBV (pM1301) Expression Vector Construction	1-24; 41-44; 50-56; 67; 71-87;	6-7-91

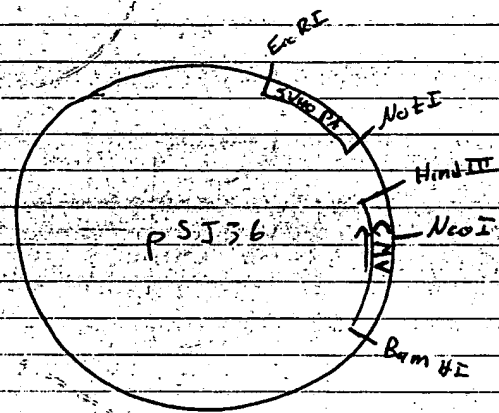
Table of Contents	Page
<u>pSJ36N<sup>-</sup></u> : NcoI destroyed in pSJ36 vector	1-7
<u>pSV42DHFR</u> : Nco deletion in SV40 Early, PCR enhancerless Promoter inserted into pSV2DHFR	8-10, 17-
<u>pSV2DHFRNco<sup>-</sup></u> : NcoI sites destroyed in pSV2DHFR X	11-16
<u>bGH PCR</u> : remove bGH poly(A) via PCR; aborted! X	21-24
<u>98-6:V<sub>K</sub></u> : PCR out 98-6 V <sub>K</sub> , subclone into pBR322	25-32, 10
<u>98-6:V<sub>H</sub></u> : PCR 98-6 Heavy chain, subclone into pBR322	33-40
<u>pSJ36NL1</u> : Kozak linker insertion into pSJ36N <sup>-</sup>	41-44
<u>pSJ36NLCAT</u> : subcloning CAT gene into pSJ36NL1	45-49
<u>pSJ36NL2</u> : Linker addition to 5' side of CMV in pSJ36NL1 X	50, 52, 67-
<u>pSV42N<sup>-</sup>DHFR</u> : Knock out of 2 NcoI sites in DHFR cassette	53, 51, 54-
<u>98-6:scFv</u> : V <sub>H</sub> V <sub>L</sub> single chain Fv of 98-6, from Batra's immunization	57-64, 96
<u>1308F/cVacFrRIP<sup>#1</sup></u> : RIP = Alt R (L4895-4) using CDS made 1308F	65-66
<u>bGH PCR #2</u> : PCR out bGH poly(A) using new primers.	71-75
<u>MIMEV-I</u> :	76-87
<u>Syd Stuff</u> : work for S. Johnson, while he was vacationing.	68-70
<u>pMEV-CAT</u> : subclone CAT gene into MIMEV vector	95-98
<u>MEV98-6HL Construction</u> : pMI304	103-113
<u>Improved MIMEV Vector</u> : aborted	115-117

TITLE pSJ36 11- Project No. 87  
Book No. 87

From Page No. pSJ36 NcoI knockout / MCS insertion  
(pSJ37 revisited)

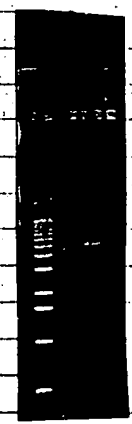
7 June 1991  
Rec'd pSJ36 DNA (500) and linkers SJ110, SJ111  
from Syd Johnson; to 40C O/N.

10 June 1991  
Cut pSJ36 & NcoI: 16.5  $\lambda$  H<sub>2</sub>O  
2.5  $\lambda$  NEB 4  
5  $\lambda$  pSJ36  
1  $\lambda$  (25U) NcoI  
- 37°C @ ~ 8<sup>15</sup> AM



SJ110 - (Hind III) NcoI NcoI SalI adapter - top  
5' (p) AgC TCC ATg gTC agC agC CgCg  
SmaI NcoI NotI  
SJ111 - bottom  
5' (p) TCC AgC Cgg CCg CCg ACC ATg g  
SmaI

Digest cut @ 10<sup>15</sup>: 170  $\lambda$  agarose / TBE gel 5RL 1Kb ladd.  
1  $\lambda$  Digest  
5  $\lambda$  pSJ36



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		Recorded by	
		<i>[Signature]</i>	11 June 1991

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TITLE PSJ3611

Page No. \_\_\_\_\_

ppL DNA (5  $\lambda$  10M  $\text{NH}_4\text{OAc}$ , 65  $\lambda$  EtOH, spin 10')

decont, dry, resuspend in 20  $\lambda$   $\text{H}_2\text{O}$ .

Fill in NcoI ends:

20  $\lambda$  DNA

2.5  $\lambda$  10X Klenow Buffer (Shirley Huan)

2.5  $\lambda$  2mM dNTPs

0.5  $\lambda$  (3 U) Klenow

37°C 35 - 45'

Add 1  $\lambda$  0.5M EDTA, 65°C 5', EtOH ppt.

Anneal linkers SJ110/SJ111 (@ 1mg/ml):

2  $\lambda$  SJ110 (2mg)

2  $\lambda$  SJ111 (2mg)

40  $\lambda$  5x BRL ligase Buffer ([MgCl<sub>2</sub>] final = 2.5mM)

1  $\lambda$  5M NaCl ([final] = 50mM)

85  $\lambda$   $\text{H}_2\text{O}$

65°C 20', remove in 400ml  $\text{H}_2\text{O}$  / 600ml beaker.

let cool to Room Temp.

Dry pSJ36NcoI filling, resuspend in 20  $\lambda$ , ligate:

19.2  $\lambda$  DNA

5  $\lambda$  5x BRL ligase Buffer

1  $\lambda$  ligase

-16°C O/N

June 1991

ppL Ligation, spin, dry, resuspend in 18.5  $\lambda$   $\text{H}_2\text{O}$ .

Cut w NcoI: 17.5  $\lambda$  DNA - 1  $\lambda$  for gel

2  $\lambda$  NEB4

0.5  $\lambda$  (12.5 U) NcoI

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D. J. S. P. Huan

Date

6/11/91

Recorded by



pSJ3611

Project No. 87

Digest @ 37°C 55 min, out @ 9<sup>00</sup>

1% agarose Gel:

- ① 250 ng H3
- ② SJ36 NeoI fill in
- ③ SJ36 NeoI fill in, religated
- ④ SJ36 NeoI fill in religated/NeoI recle

can see NeoI fill in, but nothing in the other 2 lanes.

Digest to 65°C 5', once, Xform 100ul  
Hanahan competent (J. Berlein) HB101, along c Lul pSJ  
as a control.

plate 2x100ul and 100ul 1:10 of pSJ36N (Two  
plate 100ul 1:10 pSJ36 (Two = 600ul).

37°C o/n

SJ110/III: AGC ICCAT GGT C G G C G C C G C G  
GGT ACC AG C C G C C G G C G C AG  
HindIII NeoI Not I SmaI

These adaptors have a real bad Kozak

T in the -3 position, which is the least best in that position.

-- Test SJ110/III annealing rxn.

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D. S. Pfan

Date

6/14/91

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Title

PSJ 36/11

Page No. \_\_\_\_\_

June 1991

Transformation: pSJ36 control ( $100 \lambda$  1:10 = 1.7% of transformation) gave 19 colonies.

pSJ36N<sup>-</sup>:  $100 \lambda$  (1.7% of transformation) gave 46 and 37 colonies;  $100 \lambda$  1:10 (1.7%) gave 2 colonies.

Pick 12 colonies to 4ml LB-Amp; 37°C shaker.

1<sup>st</sup>: Spin cultures, mini-preps (L.C.I.), resuspend in 50  $\lambda$  TE.

4  $\lambda$  each to 0.8% agarose / TAE gel.

note: bottom of wells must have pulled up, because sample would not enter wells very easily; lost 25%-50% of each sample.

Can't tell much, DNAs mostly running as open circles, #7, 8 look OK, all are probably ~ right size.

Inoculate 50ml cultures of 6, 7, 8; 37°C O/N.

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Date \_\_\_\_\_

Invented by \_\_\_\_\_

D. S. P. / J. A. N.

Recorded by \_\_\_\_\_

Date

6/17/91

T Page N \_\_\_\_\_

TITLE

p 5J36N

Project No.

Book No. 87

From Page No.

13 June 1991

Col 5.  $\lambda$  # 3, 4, 5, 6, 7, 8  $\Sigma$  Neo I: 5  $\lambda$  DNA1  $\lambda$  NEB43  $\lambda$  H<sub>2</sub>O1  $\lambda$  (50) Neo I-37°C @ 7<sup>30</sup> p.m.

.8% agarose/TBE Gel:

#1

#2

 $\lambda$  H3 $\lambda$  H3

5J36 Neo I

5J36 S.C.

3 S.C.

6 S.C.

3 Neo I

6 Neo I

4 S.C.

7 S.C.

4 Neo I

7 Neo I

5 S.C.

8 S.C.

5 Neo I

8 Neo I

None cut  $\Sigma$  Neo I, but that could simply be to dirty DNA, etc.

Inoculate 100ml LB-AMP cultures with # 6, 7, 8. 37°C O/N.

14 June 1991

Alkaline lysis preps of 6, 7, 8;  $\Phi$ OH/ $\text{CHCl}_3$  x1;  
 $\text{CHCl}_3$  x1, ppt 2X.

Resuspend in 200ul TE; to 4°C.

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6/17/91

Book No. 87 TITLE pSJ36N

Page No.     

7 June 1991

Cut #6, 7, 8  $\pm$  Not I, Pvu I, Not I/Pvu I:

7  $\lambda$  H<sub>2</sub>O

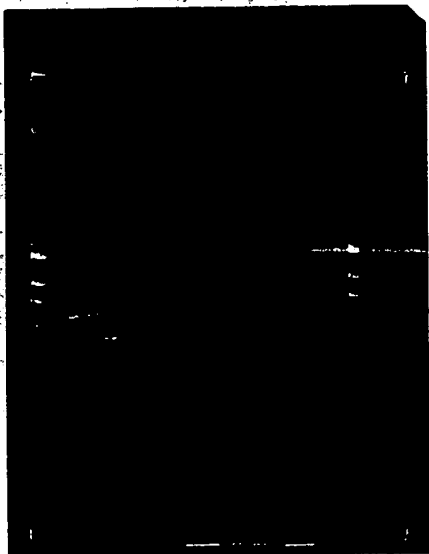
.5  $\lambda$  BRLT

.5  $\lambda$  BRLT

1.5  $\lambda$  DNA

.5  $\lambda$  Enzyme

-- 37°C @ 10<sup>20</sup> to 11<sup>20</sup>. 1% agarose/TBE:



-- pSJ36 definitely cuts  $\pm$  both enzymes.

-- #6, 7, 8 all seem to linearize with Not I, Pvu I, and Not I/Pvu I, but they don't drop out the smaller Not I/Pvu I band. (?)

-- #7 still shows mostly (all?) supercoiled in the Not I lane.

Pick #7 as pSJ36N.

June 1991 Cleanup pSJ36N #7:  $\text{NaOH}/\text{CHCl}_3$  x 2,  $\text{CHCl}_3$  x 1, pp 2.

Resuspend in 400  $\mu$ l TE, pp 2. o/N.

June 1991 Spin DNA, 70% wash, Dry, resuspend in 200  $\mu$ l TE.

Issued & Understood by me, <u>                    </u>		Date <u>            </u>	Invented by <u>                    </u>	Date <u>            </u>
			Recorded by <u>S. G. Han</u>	19 June 1991

pSJ3611-

lock no. 87

Cut pSJ3611 with *NotI*, *PvuI*, *NotI/PvuI* to assure  
*NotI* site is gone:

7.5  $\lambda$  H<sub>2</sub>O  
1  $\lambda$  DNA  
1  $\lambda$  1:1 NEB3/NEB4  
.5  $\lambda$  enzyme

- 37°C 10' out 11'

1% Agarose/TBE gel:



*PvuI* cuts in  $\beta$ -lactamase of pBR region, and  
both *NotI* and *PvuI* cut the plasmid it should  
cut into 2 smaller (~3k, 1.8k) bands.

*PvuI* does cut, *NotI* does not.

To Pa

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19 June 1991

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Book No. 7

TITLE pSV42 DHFR

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3) ~~BamHI~~ <sup>HindIII</sup> / ~~MB~~ / ~~PvuII~~ to 1% Agarose/TBE gel:

Digest looks good -- lg. fragment migrates at a lower mobility, and can see the small sub.promoter/enhancer fragments.

-- to 4°C O/N.

27 June 1991 Run DNA on 1.8% Agarose/TBE Prep gel.  
-- cut out large (4.1 kb) band. Green Clean electrophoresis O/N.

28 June 1991 spin, dry, resuspend in 50  $\lambda$  TE.  
Run 1  $\lambda$  on 1% Agarose/TBE:

DNA looks good, clean.

$\sim 50 \text{ ng}/\lambda$

July 1991 Rec'd SV40 Early promoter PCR primers:

5'  $\rightarrow$  3' = DP-P107: CCG-GAA-TTC-ACG-CGT-AGT-CCC-GCC-CC

@ 1.66  $\mu\text{g}/\mu\text{l}$  (117 pmols/ $\mu\text{g}$ ) = 194.2 pmols/ $\mu\text{l}$   $T_m \sim 81$

3'  $\rightarrow$  5' = DP-P108: GAA-GCG-CTC-GAG-CAA-AAG-CCT-AGG-CCT-CC

@ 2.54  $\mu\text{g}/\mu\text{l}$  (104 pmols/ $\mu\text{g}$ ) = 264.2 pmols/ $\mu\text{l}$   $T_m \sim 91$

dilute: DP-P107 to 2.5  $\mu\text{M}$ : 6.44  $\mu\text{l}$  (1251 pmols)  $\rightarrow$  500  $\mu\text{l}$  TE  
= 2.5 pmol/ $\mu\text{l}$

dilute: DP-P108 to 2.5  $\mu\text{M}$ : 4.73  $\mu\text{l}$  (1250 pmols)  $\rightarrow$  500  $\mu\text{l}$  TE  
= 2.5 pmol/ $\mu\text{l}$

dilute: pSV2 DHFR  $\text{M}_{60-73}$  (P/B) 1:500 in TE.

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TITLE pSV2 DHFR<sup>Neo</sup> E-

Project No. \_\_\_\_\_  
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From Page No. \_\_\_\_\_

10 $\lambda$  DNA

3 $\lambda$  5X ligase

1 $\lambda$  H<sub>2</sub>O

1 $\lambda$  (10) ligase

- RT @ 7<sup>30</sup> AM

Xform into HB101, & 1 $\lambda$  pSJ36 as control transformation,  
after expression, spin, resuspend in 17 $\mu$ l, plate 2 $\times$  of  
of pDHFR pSV2DHFR<sup>Neo</sup>.

20 June 1991. No xformants in pSV2DHFR<sup>Neo</sup> plates, very few  
on pSJ36<sup>Neo</sup> plates. Toss all.

24 June 1991. Cut pSV2DHFR and pSV2Neo with NcoI:

7.5 $\lambda$  H<sub>2</sub>O

1 $\lambda$  NEB4

.5 $\lambda$  DNA

1 $\lambda$  (250) NcoI

-- 37 $^{\circ}$ C @ 7<sup>30</sup> AM to 8<sup>30</sup> AM

Partial NcoI cut pSV2DHFR: 7 $\lambda$  H<sub>2</sub>O

1 $\lambda$  NEB4

2 $\lambda$  (4.8 $\mu$ g) DNA

.5 $\lambda$  (250) NcoI

-- 37 $^{\circ}$ C 7<sup>30</sup>; out at 8<sup>30</sup>

add 1 $\lambda$  .5M EDTA, 65 $^{\circ}$ C 5'; remove  
for gel.

1% Agarose/TBE gel:

Lane 1: pSV2DHFR S.C.

2: pSV2DHFR Nco Partial

3: pSV2DHFR Nco Complete

4: pSV2Neo Nco complete

5: X H<sub>2</sub>O

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24 June 1991

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TITLE PSV2 DHFR-Neo

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Both PSV2 DHFR and PSV2 Neo have 2 NotI sites.

PSV2 DHFR NeoI partial digest looks good.

ppz, 70% wash, dry. Resuspend in 23  $\lambda$  H<sub>2</sub>O, C.I. in:

23  $\lambda$  DNA

3  $\lambda$  10x Klenow Buffer

3  $\lambda$  10x dNTPs

1  $\lambda$  (1U) Klenow

-- 37°C 30'

ppz, dry, resuspend in 22  $\lambda$  H<sub>2</sub>O, cut with NeoI:

22  $\lambda$  DNA

2.5  $\lambda$  10x NEB4

.5  $\lambda$  (17.5U) NeoI

-- 37°C @ 4:35 PM.

5:40 PM: Add -- 17  $\lambda$  H<sub>2</sub>O

2.5  $\lambda$  10x Klenow Buffer

5  $\lambda$  10x dNTPs

.5  $\lambda$  (3U) Klenow

-- 37°C 30'

ppz. o/v.

June 1991

Spin DNA, 70% wash, dry, resuspend in 20.5  $\lambda$  H<sub>2</sub>O.

Ligate:

20.5  $\lambda$  DNA

2.5  $\lambda$  10x Ligase Buffer

1  $\lambda$  50mM ATP

1  $\lambda$  ligase (1U)

-- Room Temp 1hr.

-- add 1  $\lambda$  ligase, RT 1hr.

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Date \_\_\_\_\_

25 June 1991



Book No. 87

TITLE pSV2DHFR-11.0-

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July 1991

ppt pSV2DHFR Neo<sup>r</sup> #3, spin, dry, resuspend in 100  $\mu$ l TE.  
-- add 400  $\mu$ l Elutip-D Low Buffer, run on Elutip-D Column  
① elute, ppt, dry, resuspend in 50  $\mu$ l TE.

July 1991

cut pSV2DHFR Neo<sup>r</sup> #3 with PvuI, XbaI, PvuI/XbaI:

XbaI cuts! Toss DNA.

Already used DNA for PCR, so will subclone  
the PCR fragment and knock-out the Neo<sup>r</sup>  
site in that vector (see p. 17-18).

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7/5/91

T Page N

TITLE SV42 DHER

Book No. 87

From Page No. 10

Set up PCR reaction

- ✓ 10ul 10X PCR Buffer
- ✓ 10ul 10X dNTP
- ✓ 10ul 2.5uM DP-P107
- ✓ 10ul 2.5uM DP-P108
- ✓ 58ul H<sub>2</sub>O
- ✓ 1ul pSV2DHER Neo<sup>r</sup> 1:500
- ✓ .5ul (2.5U) Taq Polymerase

PCR File (84) 96°C/3' x 1

(86) 96°C/45", 72°C/45" x 30

(11) 8°C hold

2 July, 1991

Rxn. set at 7°C/1M; load 10ul (10%) on 2% agarose gel on P. 22

No PCR Product seen.

Setup new PCR rxn -- same as 7/1 (above)

DSP file

PCR File (84) 96°C/3' x 1

(86) 96°C/1'; 68°C/1'; 72°C/1' x 30

(11) 8°C hold

Note: T<sub>m</sub> for region of complementarity to SV40:

DP-P107: 42°C

DP-P108: 58°C

Total T<sub>m</sub> =

DP-P107 = 88°C

DP-P108 = 94°C

To Page N

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7/2/91

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Book No. 87TITLE PSVA2 DHFR

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PCR File

(84)

96°C, 3' x 1

(82)

92°C, 1' / 42°C, 1' / 60°C, 45" x 5

(86)

96°C, 1' / 66°C, 45" / 72°C, 45" x 25

(11)

80°C hold

Run 10 $\lambda$  (10%) on 20% Agarose / TAE gel:

-- 5x11 to -20°C.

SV40 promoter band looks good!

July 1991Phenol/CHCl<sub>3</sub> x1; CHCl<sub>3</sub> x1; Ppt; spin, wash, dry.Resuspend in 20.5 $\lambda$  H<sub>2</sub>O; cut  $\pm$  EcoRI:✓ 20.5 $\lambda$  DNA✓ 2.5 $\lambda$  BRL3✓ 2 $\lambda$  (100U) EcoRI

-- 37°C @ 10:25 AM; out at 1:55 PM.

-- to -20°C

2 July 1991Add: ✓ 2.5 $\lambda$  BRL3✓ 18.5 $\lambda$  H<sub>2</sub>O✓ 3 $\lambda$  (45U) EcoRI✓ 1 $\lambda$  (50U) EcoRI

-- 37°C @ 10:15 AM.

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ssed &amp; Understood by me.

Date

Invented by

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7/10/91

TITLE pSV2DHFR

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Book No. 87

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2<sup>35</sup> PM - add another 2  $\mu$ l (30U) EcoRI and  
1  $\mu$ l (50U) EcoRV  
-- to 37°C O/N

11 July 1991

pSV40A PCR RI/47III ligs. t, spin, dry, resusprn  
25  $\lambda$  TE.

Fill in ends:  $\checkmark$  25  $\lambda$  DNA

$\checkmark$  3.5  $\lambda$  10X Klenow buffer

$\checkmark$  3.5  $\lambda$  10X dNTP's

$\checkmark$  3  $\lambda$  H<sub>2</sub>O

$\checkmark$  1  $\lambda$  Klenow

-- RT @ 10<sup>00</sup> to 10<sup>40</sup> /M

-- add 1  $\lambda$  5M EDTA, 65°C 10'

Set up ligation:  $\checkmark$  4  $\mu$ l pSV2DHFR Bam.FI/19uII

$\checkmark$  5  $\mu$ l pSV40A promoter PCR RI/47III

$\checkmark$  3  $\mu$ l 10X ligase buffer

$\checkmark$  1  $\mu$ l 50mM ATP

$\checkmark$  16  $\mu$ l H<sub>2</sub>O

$\checkmark$  1  $\mu$ l Ligase

-- 16°C @ 12<sup>00</sup>

12 July 1991

Xform 12  $\mu$ l into HB101; plate on 3 plates

13 July 1991

no colonies in transformation plate.

17 July 1991

Xform 5  $\mu$ l ligation into 13  $\mu$ l ME-DH10; plate all  
2 plates

18 July 1991

Many colonies (TUTC); pick 12 to LB-amp @ 37°C

3<sup>20</sup> PM: alkaline lysis preps; to -20°C O/N.

T Pa

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Date \_\_\_\_\_

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Date \_\_\_\_\_

Recorded by \_\_\_\_\_

D. J. P. Hau

7/19/91

Project No. \_\_\_\_\_  
Book No. 87 TITLE LGH PCR Fragment

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July 1991

Rxn. out at 7<sup>00</sup> AM. Run 10ul (10%) on  
2% Agarose/TAE Gel:

No bands seen.

Setup new rxn; same as 7/1 (P21)

~~PCR File: (84) 96°C/3' x 1 DSP: 7/2  
(86) 96°C/1', 65°C/1', 72°C/1' x 3  
(11) 80°C hold~~

Note: for region of complementarity to 66H,  $T_m$ 's =

DP-P105: 50°C

DP-P106: 58°C

Total  $T_m$  =

DP-P105: 90°C

DP-P106: 96°C

PCR File: (84) 96°C, 3' x 1  
(82) 92°C, 1' / 42°C, 1' / 60°C, 45" x 5  
(86) 96°C, 1' / 66°C, 45" / 72°C, 45" x 25  
(11) 80°C soak

Run 10ul (10%) on 2% Agarose/TAE gel:

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TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. 87

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Gel on P18 -- can see ~1500 bp fragment5 July 1991

Set up PCR RXN:

- ✓ 10  $\mu$ l 10X PCR Buffer
- ✓ 10  $\mu$ l 10X dNTP's
- ✓ 10  $\mu$ l 2.5  $\mu$ M DP-P105
- ✓ 10  $\mu$ l 2.5  $\mu$ M DP-P106
- ✓ 58.5  $\mu$ l H<sub>2</sub>O
- ✓ .5  $\mu$ l D501.1 BGH-T 1:1000 (= ~1  $\mu$ g DNA)
- ✓ .5  $\mu$ l Taq Polymerase

PCR File

84  
85

94°C, 45" / 42°C, 45" / 72°C, 45" x5

86

96°C, 45" / 60°C, 45" / 72°C, 45" x25

11

8°C Soak

-- Run 10  $\lambda$  (10%) on 2% agarose/TBE!

Can possibly see a faint ~200bp band. To -20°C.

8 July 1991 $\Phi$ OH/ $\text{CHCl}_3$  x1,  $\text{CHCl}_3$  x1, ppt, resuspend in 20  $\lambda$  H<sub>2</sub>O.Tos's PCR RXN!!

DP-P105 ( <sup>5' Sal I</sup> TGC.TCG.ACG.TGC.ACG.GTC.GGT.AGA.C <sup>Rev. Pst I</sup> )  
is wrong!

-- only have a partial Sal I site and the  
Pst I site is backwards -- should be CACGTG.

Will redesign this primer and start over!!

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7/8/91

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7/8/91

To Page N \_\_\_\_\_

TITLE pSJ36N-L

Project No. \_\_\_\_\_  
Book No. 87

From Page No. \_\_\_\_\_

pSJ36N-L

8 July 1991

Kozak linker insertion into pSJ36N.

Cut pSJ36N with S<sub>1</sub>I: 20 $\mu$ l DNA  
10 $\mu$ l React 10  
65 $\mu$ l H<sub>2</sub>O  
5 $\mu$ l (5 $\mu$ l) S<sub>1</sub>I  
37°C @ 140 PM

~~Anneal DP-L101/DP-L102 linkers:~~

~~Bam HI~~

~~L101~~

~~L102~~

~~GAT CCG GTC CGT CTA~~

~~GC CAG GCAG ATCTAG~~

~~DSP~~  
~~7/8/91~~

Anneal DP-L103/DP-L104 linkers:

~~Not cutting  
Kozak over 82~~

~~Hco I~~

~~Hco I~~

DP-L103

DP-L104

AGC TAG CCG CCA CCA TGG TCG GCG GCC CG

TC GGC GGT GGT ACC AGC CGC GGG CG

✓ 1 $\mu$ l (1 $\times$ ) DP-L103

✓ 1 $\mu$ l (1 $\times$ ) DP-L104

✓ 2 $\mu$ l 10 $\times$  Ligation buffer (10 $\times$  = 5M Tris/100mM DTT/150mM NaCl)

✓ 0.5 $\mu$ l 5M NaCl [NaCl] = 100mM

✓ 20.5 $\mu$ l TE

-- 65°C 15', remove to 500 $\mu$ l 65°C bath, let cool  
-- to -20°C o/n.

Run 2 $\lambda$  pSJ36N S<sub>1</sub>I on 1% agarose/TBE: See gel P34.  
-- digest looks OK. ppt.

9 July 1991

Spin SJ36N S<sub>1</sub>I, 1 $\times$ , resuspend in 20 $\lambda$  H<sub>2</sub>O, cut  $\Sigma$  Hin

✓ 20 $\lambda$  DNA

✓ 5 $\lambda$  BRL2

✓ 24 $\lambda$  H<sub>2</sub>O

✓ 1 $\lambda$  Hind III (500)

-- 37°C @ 9<sup>00</sup> AM, out at 11<sup>30</sup> AM

To Page

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Project No. \_\_\_\_\_

Book No. 7

TITLE pSJ36NL

Page No. \_\_\_\_\_

ppt, dry, to -20°C o/n.

July 1991

Resuspend Sal/Hind III digest in 25  $\mu$ l TE, run 2  $\lambda$   
on 1% agarose/TBE:

-- can see very faint band (no picture)

-- set up ligation:

✓ 5  $\mu$ l pSJ36NL Sal/H3

✓ 2  $\mu$ l L103/L104

✓ 2  $\mu$ l 10X ligase

✓ 1  $\mu$ l 50  $\mu$ M ATP

✓ 9  $\mu$ l H<sub>2</sub>O

✓ 1  $\mu$ l ligase

-- RT incubation 7  $\mu$ l - 2  $\mu$ l

-- add 5  $\mu$ l ATP/1  $\mu$ l ligase, RT to 3

Xform 10  $\mu$ l into HB101

Cut more pSJ36NL: 25  $\mu$ l DNA

10  $\mu$ l 10X BRL10

60  $\mu$ l H<sub>2</sub>O

5  $\mu$ l (50U) Sal I

37°C @ 35, out at 44°C PM

-- 2  $\mu$ l to 1% agarose/TBE:

Cut OK.

ppt, spin, dry, resuspend in 20.5  $\lambda$  H<sub>2</sub>O

Cut with Hind III: 20.5  $\lambda$  DNA

2.5  $\lambda$  BRL2

2  $\lambda$  (40U) Hind III

-- 37°C o/n

July 1991

No colonies on transformation plates.

ppt. Sal I/Hind III digest, spin, dry, resuspend in 25  $\lambda$  TE

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7/11/91

To Page No. \_\_\_\_\_

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. 87From Page No. Setup ligation / 10  $\mu$ l SJ36N<sup>-</sup> H3/Sa1✓ 1  $\mu$ l L103/L104✓ 3  $\mu$ l 10K ligase✓ 1  $\mu$ l 50mM ATP✓ 14  $\mu$ l H<sub>2</sub>O✓ 1  $\mu$ l ligase

- - 16°C @ 11:25 AM

12 July 1991Xform 12  $\mu$ l into HB101; plate on 3 plates.13 July 1991

✓ No colonies on transformation plates

16 July 1991Xform 5  $\mu$ l of ligation mix into 13  $\lambda$  BRL AE-DH10 cells;  
on 2 plates.17 July 1991plates OK ~ 40 colonies on each. Pick 12 colo.  
to LB-Amp.18 July 1991

Alkaline lysis preps on all 12; to -20°C ON.

19 July 1991OH/CHCl<sub>3</sub> x<sup>1</sup>, CHCl<sub>3</sub> x<sup>1</sup>, pp<sup>2</sup>, resuspend in 50  $\mu$ l TECut /  $\lambda$  Nco I: ✓ 5  $\lambda$  H<sub>2</sub>O✓ 3  $\lambda$  DNA✓ 1  $\lambda$  BRL3✓ 1  $\lambda$  Nco I

- - 37°C 45'

run on .8% TAE gel

D. S. Pfau 7/19/91

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Date

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3 9 10 11 12

PROJECT NO. \_\_\_\_\_

Book No. 7TITLE pSJ36NL

Page No. \_\_\_\_\_

#1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12 all cut  $\bar{Nco}I$  and  $\therefore$  have the link  
 #4, 10 don't cut... toss these two.

Cut 10  $\lambda$  #1  $\bar{Nco}I$ : 10  $\lambda$  DNA

5  $\lambda$  BRL333  $\lambda$  H<sub>2</sub>O2  $\lambda$   $\bar{Nco}I$ 

37°C 1.5 hr; run 2  $\lambda$  on 8% Agarose/TBE:  
 digest OK; ppt O/N.

2 July 1991

Spin  $\bar{Nco}$  digest, resuspend in 25  $\mu$ l, self-ligate.

25  $\lambda$  DNA3  $\lambda$  10x ligation buffer1  $\lambda$  50mM ATP1  $\lambda$  ligase

16°C @ 950 rpm

-- to eliminate the possibility  
 of multiple linkers.

Cut pSJ36NL #1  $\bar{Nco}I$ ,  $\bar{Nhe}I$ ,  $\bar{Not}I$ ,  $\bar{Sma}I$  (all in linker); run on  
 1% Agarose/TBE gel:

All 4 enzymes cut!

innoculate 200ml culture.

3 July 1991

Quantify prep of DNA; resuspend in 200  $\mu$ l TE:

-- 470 ng /  $\lambda$  --

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Date

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7/23/91

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Project No. \_\_\_\_\_

Book No. 7

TITLE pSJ36NL?

Page No. \_\_\_\_\_

pSJ36NL2

Add the 5' polylinker to the pSJ36NL plasmid.

July 1991

Cut SJ36NL & BamHI: 1.5  $\mu$ l (2.35  $\mu$ g) pSJ36NL

✓ 3  $\mu$ l BRL3

✓ 2  $\mu$ l H<sub>2</sub>O

✓ 1  $\mu$ l BamHI (10U)

--37°C @ 8<sup>30</sup> AM

Run 1.5  $\mu$ l (118ng) on 1% Agarose/TBE:

Anneal L101/L102: 1  $\mu$ l (18) DP-L101

1  $\mu$ l (18) DP-L102

✓ 2  $\mu$ l 10x ligase Buffer

✓ 0.5  $\mu$ l 5M NaCl [NaCl]<sub>total</sub> = 100mM

✓ 20.5  $\mu$ l TE

--65°C 15', remove in 400  $\mu$ l 65°C beaker, let cool to RT.

QoHCl<sub>3</sub> Bam digest, CHCl<sub>3</sub> x1, ppt, resuspend in 20  $\mu$ l TE.

Set up ligation: 8  $\mu$ l pSJ36NL Bam/CTAP

✓ 1  $\mu$ l L101/L102

✓ 2  $\mu$ l 10x ligase

✓ 1  $\mu$ l dTP

✓ 7  $\mu$ l H<sub>2</sub>O

✓ 1  $\mu$ l Ligase

--16°C o/n

5 July 1991

ligation to -20°C o/n

7 July 1991

xform 5  $\mu$ l into competent HB101; on 3 plates

10 July 1991

Plates to 4°C -- by C. Schmidt

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Recorded by

Date

7/31/91

To Page No. 52

TITLE PSJ36UL / pSV22N-DHER

Project No.             
ok No. 37

From Page No. (P54)

31 July 1991

Pick 12 colonies to 5ml LB-amp; 37°C @ 92°/μ  
-- 2:15 PM: mini alkaline preps; resuspend DNA in 100λ  
OOH/CHCl<sub>3</sub>, CHCl<sub>3</sub>, ppt, resuspend in 50λ TE.

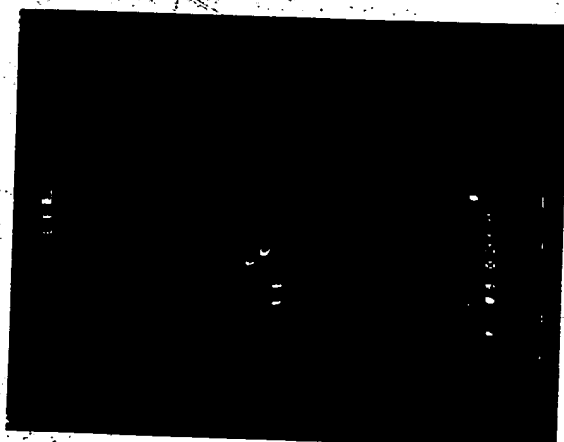
1 August 1991

Cut 3μl each of NcoI, PvuI, and NcoI + PvuI; run on  
.8% agarose/TAE: (load = Supercoiled)  
NcoI  
PvuI  
NcoI/PvuI

pSV22ADHER NcoI = ~4000bp, 800bp (sites at ~700, 4700)  
NcoI/PvuI = 2154bp, 1846bp, 800bp (PvuI at ~2854)

NcoI deletion: in SV<sub>40</sub> - ~2954  
~1846

in DHER - ~2646  
2154



#1, 2, 4, 6 have the NcoI  
deletion in the DHER reg.

#3, 5 have the NcoI del  
in the SV<sub>40</sub> early region.

Cut 20λ #3 with NcoI; 50λ d

-- 3λ to .8% agarose/TAE:

ppt. o/v.

Witnessed & Understood by me,

Date

Invented by

Recorded by

JS. P. Hall

Date

8/1/91

Project No. \_\_\_\_\_

Book No. 7TITLE pSJ36NL?Page No. 5031 July 1991

pick 12 colonies to 5ml LB-amp @ 8<sup>00</sup> AM; 2<sup>15</sup> PM -- mini prep  
 OOH/CHCl<sub>3</sub>; CHCl<sub>3</sub>, ppt, resuspend in 50  $\lambda$  TE.

August 1991

Cut 3  $\lambda$  #1, 2, 3, 4, 5, 6 with Bam and Xba I; to .8% agarose/TAE;  
 -- load SE, Bam, Xba.

Linker	g GATCCGGTCCGTCTAGg	tcc
(in CHCl <sub>3</sub> )	c c t a g G C C A G G C A G A T C T A G g	
	Bam HI	Xba I

all cut once - Bam, not with Xba I -- loss.

Cut 7-12 - Bam and Xba I, run on  
 .8% agarose/TAE as above.

Again, now have the linker  
 -- loss all.

August 1991

Phosphatase SJ36NL Bam HI: 12  $\lambda$  DNA

5  $\lambda$  H<sub>2</sub>O2  $\lambda$  10x CIAP Buffer11  $\lambda$  CIAP

-- 37°C 30', add 1  $\lambda$  CIAP -- 37°C 30'

OOH/CHCl<sub>3</sub> x1, CHCl<sub>3</sub> x2, ppt.

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TITLE pSV42N-DHFR

Project No. \_\_\_\_\_

Lab No. 87

From Page No. \_\_\_\_\_

pSV42N-DHFR

Want to knock out the *NotI* site in the SV40 *ori* promoter region.

24 July 1991

Cut pSV42DHFR w/ *NotI*: 12  $\mu$ l DNA (7.18)

5  $\mu$ l BRL 84

832  $\mu$ l H<sub>2</sub>O

0.5  $\mu$ l (2.5U) *NotI*

-37°C @ 11<sup>00</sup> AM; out @ 11<sup>35</sup> AM

Run on 8% agarose/TAE prep gel:

good partial digest! Cut out linear band, green clean, elute in 30  $\mu$ l TE.

T<sub>4</sub> fill in ends: 130  $\mu$ l DNA

✓ 4  $\mu$ l 10X T<sub>4</sub> Buffer

✓ 4  $\mu$ l 10X dNTP's

✓ 1  $\mu$ l H<sub>2</sub>O

✓ 1  $\mu$ l T<sub>4</sub> polymerase

37°C @ 3<sup>40</sup> PM, out at 4<sup>00</sup> PM

QOH/CHCl<sub>3</sub> 1:1, CHCl<sub>3</sub> 1:1, ppb. 5' suspend in 20  $\mu$ l TE, self-ligate

✓ 5  $\mu$ l DNA

✓ 2  $\mu$ l 10X ligase

✓ 1  $\mu$ l 50  $\mu$ M ATP

✓ 11  $\mu$ l H<sub>2</sub>O

✓ 1  $\mu$ l ligase

-16°C o/n.

25 July 1991

to -20°C.

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Recorded by \_\_\_\_\_

Date \_\_\_\_\_

7/29/91

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Project No. \_\_\_\_\_

Book No. E

TITLE pSV42N-D"-R

Page No. \_\_\_\_\_

29 July 1991

Xform 5  $\mu$ l into competent HB101; on 3 plates.

30 July 1991

Plates to 4°C by C. Schmidt.

31 July 1991

Pick 6 colonies to LB-amp; to 37°C @ 800 RPM.

2:5 PM: mini alkaline preps; resuspend DNA in 100  $\mu$ l TE.

90% CHCl<sub>3</sub> x1, CHCl<sub>3</sub> x1, ppt, resuspend in 50  $\mu$ l TE.

1 August 1991

SEE P. 51 for 8/1/91 Work -- OOPS!

2 August 1991

Spin NcoI digest, dry, resuspend in 19  $\mu$ l H<sub>2</sub>O; T<sub>4</sub> Fill in:

19  $\mu$ l DNA

2.5  $\mu$ l 10X T<sub>4</sub> Buffer

2.5  $\mu$ l 10X dNTPs

1  $\mu$ l T<sub>4</sub> Polymerase

-- 37°C @ 900 RPM to 94°C 1M

90% CHCl<sub>3</sub>, CHCl<sub>3</sub>, ppt. Resuspend in 20  $\mu$ l H<sub>2</sub>O, 1  $\mu$ l to 8% Agarose

Looks good. to -20°C O/N.

Read & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Recorded by \_\_\_\_\_

S. O'Fallon

Date \_\_\_\_\_

8/2/91

To Page N \_\_\_\_\_



Exhibit 4

David S. Pfarr Notebook References for Humanized IgG Expression

Notebook	Procedure	Pages	Date
87	Immunoprecipitation of COS-1 produced H1308F (pSJ60/pSJ61)	65-66	7-31-91
95	Radiolabeling RSV F protein for H1308F immunoprecipitations	4-5	7-29-91
95	CHO pSJ66-4 cell line passage for C. Schmidt	33-34	12-6-91
95	H1308F expressed from Merck Sharp and Dohme vectors in 293 cells	36-37	1-6-92
95	H1308F-R-S transfection in COS-1 cells	38-46	1-23-92
95	H1308F-R-S71 transfection in COS-1 cells	53-56	2-10-92
95	H1308F-66-71 transfection in COS-1 cells	61-65	2-27-92
95	1308F quantitation from COS-1 pSJ66 and COS pSJ60/pSJ61	100-102	9-10-91
95	H1308F purification from COS-1	103-106	10-2-91
95	H1308F purification from COS-1	107-108	10-4-91
95	H1308F purification from COS-1	110-111	10-7-91
95	H1308F purification from COS-1 pSJ66 and COS-1 pSJ60/pSJ61	115-117	10-9-91
95	H1308F purification from COS-1	119-122	10-18-91
95	H1308F purification from COS pSJ66 and COS pSJ60/pSJ61	123-127	10-22-91
95	H1308F purification from COS pSJ66	129-131	11-6-91
95	H1308F purification from CHO pSJ66	133-149	1-10-92
95	H1308F-R-S71 purification from COS-1	150-152	3-4-92
95	Western Blot: H1308F, H1308F-66-71, H1308F-S71	156-157	4-7-92
95	H1308F COS-1 supernatant analysis	171-172	9-12-91
95	H1308F COS-1 supernatant analysis	173-175	9-16-91
95	H1308F COS-1 supernatant analysis	176	9-20-91
95	H1308F COS-1 supernatant analysis	177	9-30-91
95	ELISA: purified H1308F 8-9-91	178	10-7-91

LE 1308F/rVacFr IP #1

Project No. \_\_\_\_\_  
Bo No. 87

Page No. \_\_\_\_\_

1308F/rVacFr RIP #1

C. Schmidt infected COS cells = SJ60/SJ61 (1308F Humanized H1) and I infected BSCI = RSV-F vaccinia (Book 95, p 4-5). We want to see if the 1308F is expressed in COS, and if it is we want to know if it binds to RSV-F protein.

31 July 1991

① Set up IP's (in microfuge tubes):

	Culture Super (ml)	Vaccinia Lysozyme
SJ60/SJ61 #1	.4 ml	.2 ml F / .15 ml TK <sup>-</sup>
SJ60/SJ61 #2	.4 ml	.2 ml F / .15 ml TK <sup>-</sup>
Mock #1	.4 ml	.2 ml F / .15 ml TK <sup>-</sup>
Mock #2	.4 ml	.2 ml F / .15 ml TK <sup>-</sup>

-- rotate @ 4°C: 1<sup>h</sup> --

1308F (10 ng/ml) #1	1 ml / 400 μl PBS	.2 ml F / .15 ml TK <sup>-</sup>	} 4°C @ 24h
1308F (10 ng/ml) #2	1 ml / 400 μl PBS	.2 ml F / .15 ml TK <sup>-</sup>	

② 4<sup>h</sup>: Add 100 μl Activated Staph A to each tube, on ice 10'

-- only enough Staph A cells for 11 samples; less 1308F / TK

- Spin 5K, 5', aspirate
- resuspend in 200 μl 1M Buffer, vortex & triturate
- Spin 5K, 5', aspirate
- resuspend in 200 μl RIPA
- Spin 5K, 5', aspirate
- Resuspend in 200 μl RIPA, transfer to new tube.
- Spin 5K, 5', aspirate
- to -20°C o/n

To Page No. \_\_\_\_\_

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

7/31/91

Project Nr

Book No. 87TITLE 1308F/r Vac Fr RIP #1Page No. 11 August 1991Resuspend pellets in 50  $\mu$ l SDS loading dye; boil 7!load 25  $\mu$ l each onto 4-20% Proteom II gradient gels;

Gel #1		Gel #2	
Lane	Sample	Lane	Sample
1	Rainbow Samples	1	—
2	Mock 1 - F	2	Rainbow Standards
3	Mock 1 - TK	3	Mock 2 - F
4	SJ60/61 - F	4	Mock 2 - TK
5	SJ60/61 - TK	5	SJ60/61 - F
6	1308F - F	6	SJ60/61 - TK
7	1308F - TK	7	1308F2 - F
8	—	8	—
9	—	9	—
10	—	10	—

dry gels, on film @ 4:00 PM.

1 August 1991

Develop film. Can barely see F protein in 1308F control lane, not in other lanes.

New film on @ 2:00 PM.

1 August 1991Develop film, 9:00 AM. Can definitely see F protein put down by 1308F control (10  $\mu$ g) and in the SJ60/61 lanes, but better in gel #2 than gel #1.

Witnessed &amp; Understood by me,

Date

Invented by

Date

To Page No. 1

Project No.

Book No. 5

TITLE rVAC-Fr

Page No. 1

rVAC-Fr Labeling

Want [25S]-Met labeled RSV F protein to use in RIP assay of 1308F antibodies.

9 July 1991

Rec'd. 4 P100s of BSC1 cells from C. Schmidt, split 1:20 on 7/26/91.

① Thawed 2 virus stocks: rVAC-Fr  $1.7 \times 10^{10}$  PFU/ml  
vSH.CS #4  $2 \times 10^{10}$  PFU/ml -- TK<sup>-</sup> vaccinia expressing malaria CS protein

-- Sonicate 3 x 10 sec, in cup etc., setting 8.

② Cells should be  $\sim 8 \times 10^6$ /plate; want an MOI of 10 with the inoculum in 1.5 ml medium (EMEM/2.5% FBS/L-Glu/NaPy/P/S)

-- rVAC-Fr: 8 ml/3 ml, for 2 plates

-- vSH.CS #4: 8 ml/3 ml, for 2 plates

Aspirate medium from plates, add 1.5 ml inoculum, to 37°C.

③ Rock plates every 15' for 1.5 hr. -- 10<sup>00</sup> to 11<sup>00</sup>

④ Add 8.5 ml medium, to 37°C, for 2.5 hr: 11<sup>00</sup> - 2<sup>00</sup>

-- 3 plates (one rVAC-Fr and both TK<sup>-</sup>) were too sparsely populated -- they were seeded lightly (1:20) on 7/26 for use later this week.

-- Toss three light plates, one rVAC-Fr allowed to continue

-- Repeat steps 1-4 with 3 new plates, one rVAC-Fr, 2 vSH.CS #4

① inoculum on at 2:45 PM.

② add 8.5 ml medium @ 4:15 PM.

make medium: 5.44 ml DMEM w/o Met

105 ml each: L-Glu, NaPy, P/S

1.4 ml DMEM complete + 10% FBS

Final

Page No. 1

Witnessed & Understood by me,

Adrian D

Date

11/12/91

Invented by

Adrian D

Date

11/12/91

TITLE rVacFr #1

ject No. \_\_\_\_\_

Book No. 95

From Page No. \_\_\_\_\_

(C) 5<sup>45</sup> PM: Scrape cells off plates, transfer to 50ml tubes (combine rVacFr cells); spin 2K, 5'.

(D) aspirate medium, resuspend in 5ml DMEM w/o Met, spin.

(E) aspirate medium, resuspend:

(1) VSH.CS #4 in 1.5ml labeling medium w/o [<sup>35</sup>S] / TK<sup>-</sup> Cold

(2) VSH.CS #4 in 1.5ml labeling medium, add 45ul [<sup>35</sup>S] Met @ 10.6 uCi/ml = 477 uCi

(3) resuspend rVacFr in 3ml labeling medium, add 90ul [<sup>35</sup>S] Met = 954 uCi

- Note: [<sup>35</sup>S] has already gone through 1st Met and only adding 1/2 as much label as normal.

(F) to 37°C O/N.

30 July 1991

-- by Tom Furst:

\* Spin cultures, aspirate sups, resuspend in RIPA

(1) rVacFr: 2ml RIPA

(2) VSH.CS #4 TK<sup>-</sup> Hot: 1ml RIPA

(3) VSH.CS #4 TK<sup>-</sup> Cold: 0.5ml RIPA

Vortex vigorously, to -20°C.

31 July 1991

Thaw lysates, spin 30K, 30min, 4°C SW50.1 rotor

Remove sups to 3ml tubes; cleared lysates.

Witnessed & Understood by me,

and 11/11

Date

11/11

Invented by

Tom Furst

Date

11/11

To Page

11  
TITLE SJ66-4  $10^{-8}$  MTX and  $10^{-7}$  MTX Maintenance Project No. 98  
book No. 95

From Page No. SJ66-4  $10^{-8}$  MTX and  $10^{-7}$  MTX

6 December 1991

Harvest cells, resuspend in 10ml, count:

SJ66-4  $10^{-8}$  MTX:  $4.5 \times 10^5$  cells/ml  $\times 10$  ml

-- Seed 1 flask @  $4 \times 10^5$  cells/ml  $\times 10$  ml = (P1)

SJ66-4  $10^{-7}$  MTX:  $6.0 \times 10^5$  cells/ml  $\times 10$  ml

-- Seed 1 flask @  $4 \times 10^5$  cells/ml  $\times 10$  ml = (P8)

9 December 1991

T75's look stressed -- cells with drawing and many floaters

Harvest cells, count:

SJ66-4  $10^{-8}$  MTX

$6.0 \times 10^5$  cells/ml  $\times 10$  ml, 100% viable

-- Seed 1 T75 @  $2 \times 10^5$  cells/ml  $\times 10$  ml = (P11)

SJ66-4  $10^{-7}$  MTX

$5.5 \times 10^5$  cells/ml  $\times 10$  ml, 87% viable

-- Seed 1 T75 @  $2 \times 10^5$  cells/ml  $\times 10$  ml = (P9)

-- Seed remaining cells into a T150 in 35ml medium.

13 December 1991

Harvest T75's, count:

4 day {  
SJ66-4  $10^{-8}$  MTX:  $5.7 \times 10^5$  /ml  
SJ66-4  $10^{-7}$  MTX:  $9.2 \times 10^5$  /ml

Harvest T150's seeded 12/9, pool cells with T75 cells,  
Count:

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To Page 1

Project No. 40

Book No. 5

TITLE SJ66-4  $10^{-8}$  and  $10^{-7}$  MTX

m Page No.     

SJ66-4  $10^{-8}$  MTX:  $2.54 \times 10^6$  cells/ml  $\times 13$  ml

SJ66-4  $10^{-7}$  MTX:  $2.71 \times 10^6$  cells/ml  $\times 13$  ml

-- Seed 1 spinner each @  $2 \times 10^5$ /ml  $\times 100$  ml

December 1991

SJ66-4  $10^{-8}$  MTX spinner contaminated w/ bacteria; Toss

Count SJ66-4  $10^{-7}$  MTX:  $7.8 \times 10^5$ /ml, 91% viable -- many clump  
cells in "strings" that couldn't be counted.

Spin 50 ml, resuspend in 25 ml, count:  $9.2 \times 10^5$ /ml

-- Seed ~~1~~ spinner @  $2 \times 10^5$ /ml  $\times 100$  ml

December 1991

Cell count:  $6.6 \times 10^5$ /ml, 53% viable

Spin 70 ml, resuspend in 25 ml:  $1.26 \times 10^6$ /ml, 60% viable

Seed 19 ml  $\rightarrow$  120 ml spinner:  $2 \times 10^5$ /ml  $\times 120$  ml

Obviously leaving cells in this medium for  $> 3$  days is  
quite detrimental to their health. Until some medium  
development gets done I won't be able to grow cultures  
productively for more than 72 hrs.

3 December 1991

Cell count:  $4.5 \times 10^5$ /ml, 85% viable Cells quite clumped  
and aggregated! Other cell lines are coming along that  
are much better.

Toss cells.

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8/5/92

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*D. S. R. / 1000*

Date

2/23/91

Project No. 03.95

Book No. 15

TITLE 293 Transfection 447D, 1308F

Page No.

293 Transfection

1308F and 447D cotransfections

I want to compare the productivity of the Muckevector/293 transient system versus the SV40ori/COS system to see which gives higher yields of protein. Colleen Schmidt will do the COS transfection in parallel with my 293 transfect

January 1992

Harvest 293 cells, resuspend in 20ml (LNB95-23); count:

$1.72 \times 10^6$  cells/ml

Seed 7 plates  $\approx$  2.3ml cells ( $4 \times 10^6$  cells/plate) in 12ml Erlen.

January 1992

Prep DNA for transfections:

1308F

PM1-1308FH4 @ 0.57  $\mu$ g/ml - 70  $\mu$ l = 40  $\mu$ g } combine and ppt  
PM3-1308FL6 @ 0.49  $\mu$ g/ml - 82  $\mu$ l = 40  $\mu$ g } o/n

447D:

H1447D @ 1.08  $\mu$ g/ml - 37  $\mu$ l = 40  $\mu$ g } combine and ppt  
L3447D @ 0.725  $\mu$ g/ml - 55  $\mu$ l = 40  $\mu$ g } o/n

January 1992

Spin DNAs, 70% wash, air dry in hood.

① Resuspend DNAs in 1ml TBS each

② Add DNA/TBS to 4ml DEAE-Dextran/Chloroquine/DNEM

③ Wash 6-P100's  $\approx$  PBS, add 2.5ml DNEM/10% HUS serum per plate.

④ add 2.5ml DNA/DEAE-Dextran to each of 2 plates for 1308F, 447D, 1

⑤ 6hrs later - DMSO shock cells (10% DMSO/PBS)

- cells lifting off plate in DMSO shock, lost  $\sim$  30%-50% of cells.

⑥ Add 15ml medium to each plate.

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TITLE 293 Transfection: 147D + 1308F

Project No. 83.95

Book No. 95

From Page No.     

10 January 1992

4<sup>00</sup> PM: Harvest medium from plates, spin, decant, resusp  
cell pellets in 15ml medium. Return to plates.

- Save 1ml aliquot from each plate for assay, pool remaining  
media, store @ -20°C.

13 January 1992

4<sup>00</sup> PM (72 hrs) Harvest medium, separate samples from each  
plate for assay, pool like medium. To -20°C.

Toss plates -- cells ~90% sloughed off.

14 January 1992

ELISA: No Ab seen in any of the samples in  
G&H IgG ELISA.

B7 down ELISA: No Ab detected binding to the B7 peptide.

-- The DEAE-Dextran transfection of the 293 cells obvi  
did not work, since parallel transfections into COS  
cells (C15) did work. Will either have to work out the  
conditions for DEAE-Dextran mediated transfection of 293 c  
or use another transfection method.

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Book No. 5

TITLE COS: H1308F → S, H1129611, CH1129611

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## Cos Transfection

CH129b/Ch129e: Chimeric 1129

H11296/H11292: Humanized 1129

3 January 1992

Co-precipitate DNAs for 2-60mm dishes for each transfection:

H1308FE/H1308FH: R-45

SJ61 1.72 mg/ml 11.6 ml

R-5#1 = 0.67 mg/ml : 30  $\mu$ l

$R \rightarrow S^{\circ} 2 = 0.36 \text{ mg/l} \therefore 56 \text{ ml}$

Ch1129h/Ch1129e

$\rho_{SJ75} = 0.82 \text{ mg/ml} \therefore 24 \text{ ml}$

ch11292 d1 = 0.25 mg / ml : 80 ml

ch 1129L-2 = 1 bag full : 20 ml

H 11296 / H 11298

$H_{1129h} = 5.34 \text{ g/l} \quad 3.7 \text{ ml}$

H1129L #1 = 0.32 inch : 63 all

H11292  $z = 0.4$   $\mu\text{g}/\text{ml}$ : 50  $\mu\text{ml}$

Humanized and chimeric light chain  
tubes are mislabeled.

Save these for later  
CH/H mixing experiments

4 January 1992

Ca Precipitate more  $\text{NH}_3$ :

CN11296 CH11290

$\rho_{5575} = 0.82 \text{ mg/ml} = 24 \text{ ml}$

CH11292<sup>vi</sup> = 0.32 mg / ml : 63 ml

CH 1129202 = 0.4 mg/ml: 50 ml

H1129H / H1129L

$H_{1129h} = 5.34 \mu g/l$

H112221 = 0.25mg/ml : 80ml

H1129202 = 1 ng / ml : 20 ml

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2/17/92

DATE: 11/2/11

1/16

Project No. 98

Book No. 95

TITLE COS: H1308Fh; S; H1129h/L; CH1129h/L

Form Page No. 1

Clean up samples for Western blot using Staph A cells (Pansorb cells):

① Activate 1ml pansorb cells:

-- Spin, decant

-- Resuspend in 1ml STE-5% NP40, RT 20'

-- Spin in microfuge, setting 5, 5'; decant

-- resuspend in 1ml STE-0.5% NP40, RT 5'

-- Spin, decant.

-- Resuspend in 8ml STE-0.5% NP40-1mg/ml Ovalbumin, store on ice.

② Bind MAb to Staph A:

-- add 1ml of each combined supernatant to 1ml Activated Pansorb cells;

-- add 1ml SJ66 Fucin #2 GPH Flow Thru #2 (LN 95-149-1187 mg/ml) to 1ml cells.

-- Rotate, 4°C, 15'; on ice 10'

③ Wash cells:

-- pellet cells, setting 5 (microfuge), 5'; decant

-- resuspend in 5ml 1M Buffer

-- pellet, decant

-- resuspend in 1ml RIPA

-- pellet, decant

-- resuspend in 15ml RIPA, transfer to aden tube

-- pellet, decant to -20°C O/N.

29 January 1992

Resuspend pellets in 100µl 1X loading buffer, boil 5', Spin 5' in microfuge, remove supes. to new tube.

Load 25µl of each sample onto 15% Disc-SDS/PAGE

Lane	Sample
1	Amersham Rainbow stds.
2	SJ66 Fucin #2 Flow Thru #2
3	Mock
4	H1308F: R-5 #1
5	H1308F: R-5 #2
6	CH 1129 #1
7	CH 1129 #2
8	H1129 #1
9	H1129 #2

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D. S. P. / M. L.

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TITLE COS: H1308FR-S; H1129h1; CH1129h1/2

Project No. 98

Book No. 95

From Page No.

- Run dye front to ~ 1 cm from bottom.
- Blot to 2mm nitrocellulose: 100V, 1 hr.
- PBS wash blot, dry 2/2.

30 January 1992

- Peroxidase block in H<sub>2</sub>O; block in TB3-5% milk 1 hr, RT.
- Wash 15' PBS-Tween, wash 2x 5' PBS-Tween
- Add 10ml G&H IgG-HRP (1:1000), incubate RT 1 hr.
- washes above, add 6ml ECL reagent, incubate 1 min, expose to film.

### Results

The IgGs are all undenatured and running at ~160kd!! Perhaps it is too long before loading the gel. A BSA band can be seen in all the lanes that have TC supernatant samples, so the IP didn't get rid of all of the BSA.

Repeat gel - boil samples

- 4 samples popped open and flooded up out being lost: both H1308 and one CH1129! Toss all samples and start over.

No Pansorbin cells left, so will use Protein A-Sepharose to immunoprecipitate samples.

① Add 1.28 mg of each Ab supernatant to a microfuge tube

Mock - 1ml

H1308FR-S#1 - .75ml

H1308FR-S#2 - .57ml

CH1129 #1 - .91ml

CH1129 #2 - 1ml

H1129 #1 - 1ml

H1129 #2 - 1ml

SJ66 Furin #2: F/wt #2 - .68ml

-- bring up to 1ml w/ DMEM/10% FCS

② Add 150ul Protein A-Sepharose to each tube, rotate 4°C, 1 hr + 15'

③ Spin 1' microfuge, decant, resuspend in 1ml PBS - .5M NaCl - 1% Triton, spin, d.

④ Repeat above wash; wash 2x in PBS-1% Triton and 2x in PBS.

⑤ Resuspend final pellet in 100ul 1.2X Sample loading buffer. To 2c

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Project No. 98Book No. 5TITLE COS: H1308F R<sup>+</sup>, CH1129; H1129Page No. 1January 1992

Boil Samples 5', spin 30" microfuge

Load 20  $\mu$ l onto 15% SDS-PAGE

Lane	Sample
1	Reinbow SDS empty
2	Reinbow SDS
3	SJ66 K <sup>+</sup> in #2 FlowThru #2
4	Mock
5	H1308F R <sup>+</sup> S #1
6	H1308F R <sup>+</sup> S #2
7	CH1129 #1
8	CH1129 #2
9	H1129 #1
10	H1129 #2

Run gel, blot to nitrocellulose, wash in H<sub>2</sub>O, dry, to 4°C O/N.

add flasks of BSC cells from SJ (T25's):

- ① infected E. Vac F virus, <sup>35</sup>S labeled
- ② in infected TK<sup>-</sup> virus, <sup>35</sup>S labeled
- ③ uninfected, <sup>35</sup>S labeled

- Harvest cells, PBS wash, resuspend in 1ml RIPA, Spin 30k, 30', SW55T.
- Remove Supers to clean tubes, -20°C O/N.

February 1992

Rehydrate Blot, block in 5% Milk-TBS RT, 1hr.

Probe E. G $\alpha$ H IgG-ABP, 1:1000, RT 1hr.

Wash, ECL detect, expose to film

Results

H1308F R<sup>+</sup> S #1 and #2, and CH1129 #1 and #2 <sup>OSP</sup> all show good amounts of Ig $\gamma$  -- both heavy and light chain without a gross excess of light chain. Neither H1129 shows any Ig $\gamma$  -- heavier light chain corroborating the results seen in the ELISA.

To Page No. 2

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2/17/92

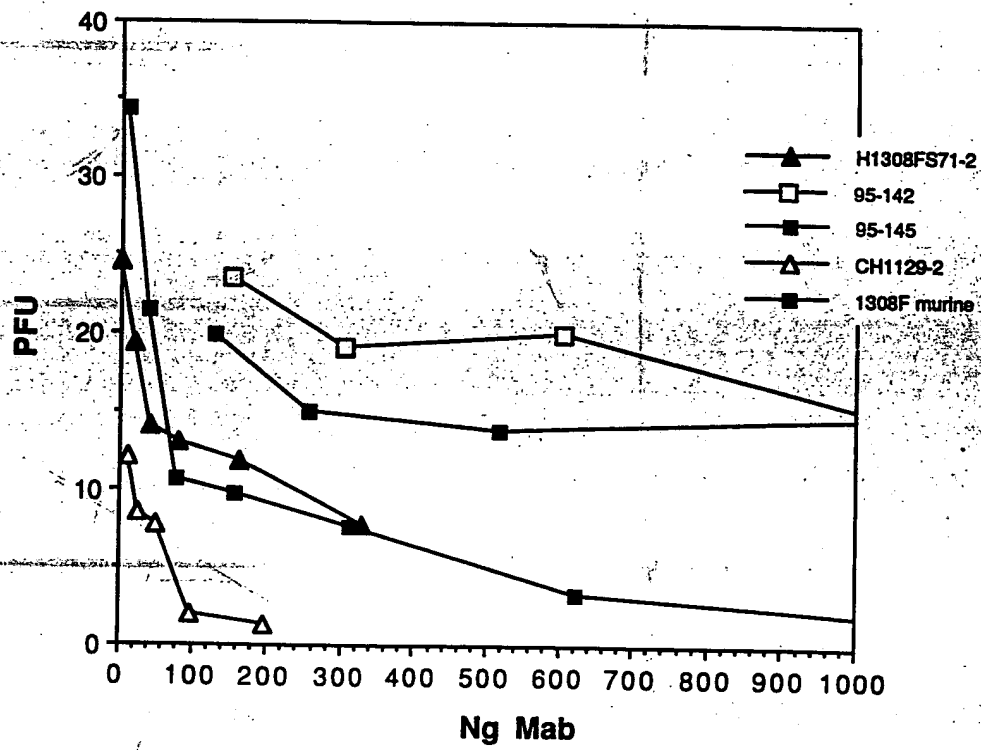
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D. J. S. P. H. A. W.

Date

2/3/92

# RSV Plaque Reduction Assay



TITLE COS: H1308F R+S; CH1129; H1129

Project No. 98

Book No. 95

From Page No.

Note: Transfection plates were harvested again on 1/30/92; the cells were also b and the pellets frozen away.

ELISA Results	1/24 → 1/27	1/27 → 1/30	
H1308F:R+S #1a	1.66 µg/ml	6.30 µg/ml	
H1308F:R+S #1b	1.75 µg/ml	4.32 µg/ml	
H1308F:R+S #2a	2.63 µg/ml	7.62 µg/ml	Combined sample re-assayed 2/9/92: 5.41 µg/ml
H1308F:R+S #2b	1.86 µg/ml	5.50 µg/ml	
CH1129 #1a	1.23 µg/ml	5.84 µg/ml	Samples are all ~4.5 ml each.
CH1129 #1b	1.59 µg/ml	5.34 µg/ml	
CH1129 #2a	1.34 µg/ml	4.45 µg/ml	
CH1129 #2b	1.22 µg/ml	3.43 µg/ml	Combined sample re-assayed again 2/11/92: 3.27 µg/ml DS
H1129 #1a	-0- µg/ml	ND	
H1129 #1b	-0- µg/ml	ND	
H1129 #2a	-0- µg/ml	ND	
H1129 #2b	-0- µg/ml	ND	
Mock 1	-0- µg/ml	-0- µg/ml	
Mock 2	-0- µg/ml	0.17 µg/ml	

## RIP

Need to see that both H1308F:R+S and both CH1129's still bind to the F protein of

### ① Pre-Adsorb Vac. lysates

-- add .5ml 1/4-1/2 Mock COS sups to each lysate (1/4µg), rotate 1.5 hrs @ 4°C.

-- add 100µl Panarbin cells to lysates, rotate 4°C 30', spin, remove sups to new tube.

### ② Bind Sups (1/4-1/2) to lysates

-- Add 2nd lysate to .5ml COS sups from 1/4-1/2; rotate 1 hr @ 4°C.

### ③ Wash Adsorb to step 1

-- add 100µl Panarbin cells to each sup, rotate 1 hr @ 4°C.

-- spin 5' @ 500g, Biofuged, decant

-- wash E .5ml 1M Buffer, spin, decant, repeat spin wash

-- wash E .5ml RIP, spin, decant, repeat wash; transfer to fresh tube, spin, dec

-- Resuspend in 100µl 1.2X Sample loading buffer.

### ④ Load 25µl onto 15% SDS-PAGE

-- boil samples 5'

-- spin 12k, biofuge, 5'

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2/1/92

Project No. 98  
Book No. 5

TITLE COS: H1308FR-5; C. 29; H1129

n Page No.       

Gel #1

Lane 1 - rainbow markers

2 - Mock/Flyate

3 - Mock/TK-lyate

4 - Mock/uninfected lysate

5 - H1308FR #1/Flyate

6 - H1308FR #1/TK-lyate

7 - H1308FR #1/uninfected lysate

8 - H1308FR #2/Flyate

9 - H1308FR #2/TK-lyate

10 - empty

Gel #2

Lane 1 - rainbow markers

2 - Mock/Flyate

3 - Mock/TK-lyate

4 - Mock/uninfected lysate

5 - CH1129 #1/Flyate

6 - CH1129 #1/TK-lyate

7 - CH1129 #1/uninfected lysate

8 - CH1129 #2/Flyate

9 - CH1129 #2/TK-lyate

10 - empty

Run gels, soak 1 hr. in 1M NaSalicylate, dry, on film at 8<sup>30</sup> PM.

During washes, realized that I hadn't activated the Penicillin cells! Repeat RT

① Spin 5ml Penicillin cells, resuspend in 5ml STE-5%NP40, RT 15'

② Spin cells, resuspend in 5ml STE-0.5%NP40

③ Spin cells, resuspend in 5ml STE-0.5%NP40 - include Ovalbumin

④ Bind Supers to lysates

... add 0.1ml lysate (TK and Vact) to .25ml COS Supers, rotate 1 hr @ 4°C.

⑤ Add 50 $\mu$ l Activated Penicillin cells to Supers/lysates; rotate 30' 4°C.

⑥ Wash cells as above (LUB 95-43), use .2ml volumes

⑦ Store final gels @ -20°C.

February 1992

Resuspend RIP #2 pellets in 50ml 1.2X Sample Buffer, boil 5', spin in microfuge 5', load 20ml on 15% SDS-PAGE:

Gel 1

Lane 1 - Blank

2 - [<sup>35</sup>S] Rainbow Markers

3 - Mock/Flyate

4 - Mock/TK-lyate

5 - H1308FR #1/Flyate

6 - H1308FR #1/TK-lyate

7 - H1308FR #2/Flyate

8 - H1308FR #2/TK-lyate

9 - Blank

Gel 2

Lane 1 - [<sup>35</sup>S] Rainbow Markers

2 - Blank

3 - Mock/Flyate

4 - Mock/TK-lyate

5 - CH1129 #1/Flyate

6 - CH1129 #1/TK-lyate

7 - CH1129 #2/Flyate

8 - CH1129 #2/TK-lyate

9 - Blank

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-- COS: H1308F R+ S, CH1129, 11129

Exp No. 98

Book No. 95

-- Run gel, fix in destain 30', wash 5' in  $H_2O$ , soak in 1M NaSalicylate 30'  
On gel @ 5<sup>20</sup> PM.

RIP #1: develop gel @ 7<sup>20</sup> AM (11 hr exposure): very faint; can see 3 lanes in all infected lanes but not in uninfected lanes; so these are obviously Vaccinia lanes. Can see F protein in both H1308F/VacF lanes and in both CH1129/VacF lanes. Both H1308F R+ S and both CH1129 molecules bind to RSV-F protein.

-- put new film on blot @ 7<sup>20</sup> AM.

5 February 1992

Develop RIP #2 film at 8<sup>20</sup> AM (15 hr exposure): very clean gel. Can see RSV-F bands in all 4 H1308F R+ S and CH1129 lanes; very light exposure.

-- put new film on gels at 8<sup>20</sup> AM

6 February 1992

Develop RIP #1 film -- 55 hr exposure

- ① There are ~7 major background bands that are attributable to the cells/Vaccinia system.
- ② Can see both RSV-F bands only in the H1308F R+ S and CH1129 lanes with the F lysates. All four constructs are producing functions!

7 February 1992

Develop RIP #2 film, 55 hrs:

- ① Very clean -- can see RSV-F bands only in the H1308F R+ S and lanes with VacF lysates; No other background bands are really visible. Apparently activating the StaphA cells does not increase the capacity/ability to bind IgS, but it does reduce the background.

10 February 1992

2.5 ml each of: 1/30 Mock

1/30 CH1129 #2

1/30 H1308F S-71 #2

Additional 2.5 ml to  
CHR 2/18/92 for Neut ass

given to F. Brady and J. Tamura

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2/11

Project No. 98

Book No. 12

TITLE COS: H1308F571, -H1129; H1129

Page No.     

2 February 1992

Ran RIP against <sup>35</sup>S-F protein (LNR95-49) using all the proper (avoid  
+ and - controls. Very light exposure shows that CH1129 <sup>92</sup>(430) pulls down  
specifically the F protein.

3 February 1992

50 hr. exposure of RIP shows very clearly that the RSV-F protein is  
immunoprecipitated with the CH1129.

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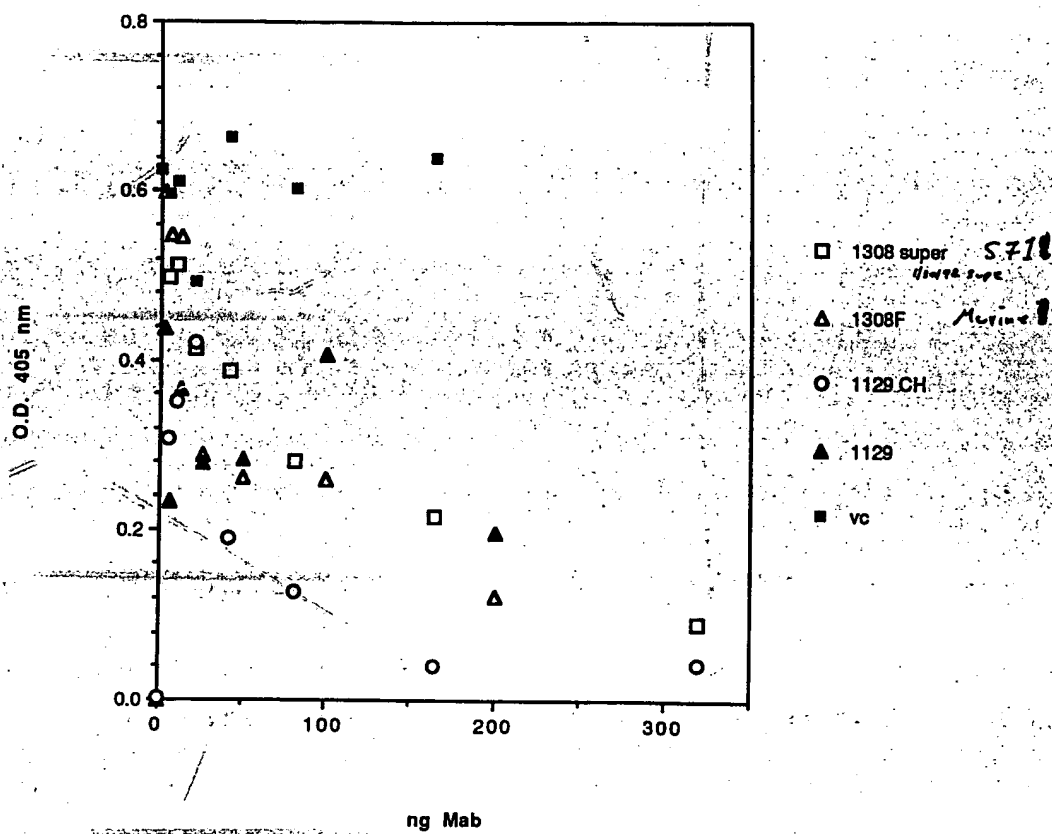
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Page No.

2/14/92

# RSV Microneutralization



|||

TITLE COS: H1308F-S71; CH1129; MEV98-6H/L; H1129h/L

Project No. 98/85

Book No. 95

From Page No. \_\_\_\_\_

# COS Transfection

H1308F-S71 and CH1129 and MEV98-6H/L and H1129h/L

Want to produce more H1308F-S71 (formerly H1308F-R-S #2) and CH1129 (#2)  
Further analysis; want to see if MEV98-6H and MEV98-6L express their respective

10 February 1992

Harvest COS1 cells, count:  $2.7 \times 10^6/\text{ml} \times 20\text{ml}$

Seed 10 P100s at  $3 \times 10^6$  cells/plate.

11 February 1992

Precipitate DNAs:

(1) H1308F-S71 #2

-- 96  $\lambda$  pSJ61 (80x)

-- 111  $\lambda$  H1308F-S71 #2 prep 1 (40x)

-- 63  $\lambda$  H1308F-S71 #2 prep 2 (40x)

ppb, 70% wash, resuspend  
in 2ml TBS

(2) CH1129 #2

-- 69  $\lambda$  pSJ75 (56x)

-- 242  $\lambda$  CH1129 #2 prep 2 (62x)

ppb, 70% wash, dry, resuspend  
in 2ml TBS

(3) 98-6

-- MEV98-6H - 20  $\lambda$  (20x)

-- MEV98-6L - 20  $\lambda$  (20x)

ppb, 70% wash, dry, resuspend  
in 0.5ml TBS

DEAE/Dextran transfect as usual, 4 plates = H1308F-S71, 3p  
= CH1129, 1 plate with 98-6, 1 plate = H1129h/L #1, 1 plate =  
H1129h/L #6.

-- H1129h: 3.7 ml (20x) for each xfection.

-- H1129L #1 (2.11.92, SJ): 2.9 ml (20x)

-- H1129L #6 (2.11.92, SJ): 2.5 ml (20x)

-- DNA on cells 6.5 hrs. before DMSO shock.

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Project No. 98/83  
Book No. 15

TITLE COS: H1308F-S71; H1129; MEV98-6; H1129-6

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4 February 1992

Harvest supes at 9<sup>00</sup> AM (63.5 hrs); ELISA:

repeated  
LUB95-48  
{ H1308F-S71: 3.11 µg/ml x 38 ml  
CH1129: 4.72 µg/ml x 28.5 ml

H1129 #1: 102 ng/ml x 9.5 ml  
H1129 #6: 248 ng/ml

MEV98-6: 3.98 µg/ml

2.5 ml given to  
CHR 2/18/92 for  
Nest assays.

DSP  
2/18/92

Feed plates with 10 ml medium

-- The H1129s are over a log lower than everything else; it is a question of the accuracy of the light chains, so there may be bad constructions. Toss the H1129 #1 and #6 plates.

-- The MEV98-6 expressors, and at high levels!! The crippled SV40 early region is still retains sufficient information to allow for the replication of the plasmids. Also, the 98-6 heavy and light chains apparently have no major frame shifts since intact Ab is being produced.

-- High levels of both CH1129 and H1308F-S71 are being produced.

7 February 1992

Harvest supes from plates; feed plates & 10 ml medium.

Western: same as western LUB95-48

-- Activate Staph cells

-- Add 100 cells to:

① 1.5 ml Cos Mook supernatant

② 1.5 ml H1129 #1 (3/14): 153 ng

③ 1.5 ml H1129 #6 (3/14): 372 ng

④ 0.5 ml MEV98-6 (3/14): 1.99 µg

⑤ 1 ml 1.35 µg/ml H1gG1.5 diluted to 1 ml in PBS

⑥ 1.25 ml H1308F 95-121 fraction #2 = 1.01 µg

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D. S. Plasm

2/17/92

11  
TITLE COS: H1308F-S71; CH1129; 1-98-6 h.c.; H1129 h.c.

Project No. 98/83  
Book No. 95

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- rotate suspensions, 4°C, 45'
- pellet, resuspend in .5ml 1M buffer, pellet
- resuspend in .5ml RIPA, pellet
- resuspend in .5ml RIPA, transfer to new tube, pellet
- resuspend in 100µl 1X loading buffer.
- to -20°C OK.

18 February 1992

Boil samples 5', spin 7' in microfuge, load <sup>25µl</sup> on 4-20% Gradient  
-- See LUB95-48 for order and run time.

#### Western Results

- 98-6 seems to be running slightly fast, both heavy and light chains. This needs to be repeated.
- H1129 and #6 can be seen very faintly, however 15 fold and respectively; less protein were present in these supers. sizes to match up with the H1CH1129 bands.

#### ELISA of 2/18/92 supers:

H1308F-S71: 3.9 µg/ml  
CH1129: 5.9 µg/ml  
MEV98-6: 6.7 µg/ml

Repeats of 2/14 samples:

H1308F-S71: 2.44 µg/ml (3.1)  $\bar{x}$  =  
CH1129: 4.17 µg/ml (4.72)  $\bar{x}$  =

20 February 1992

Harvest COS supers; ELISA:

H1308F-S71: 1.97 µg/ml  
CH1129: 2.93 µg/ml  
98-6: 6.74 µg/ml

Ran RIPA against <sup>35</sup>S-F (LUB95-49): 14hr exposure shows that 98-6 does not ppt. the F protein. No F is seen in either H1129. ~~either~~ A longer exposure may be needed.

Witnessed & Understood by me,

Date

Invented by

Date

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Project No. 78/83

Book No. 45

TITLE COS-H1308F.571; L11129; MEV98-6hml; H1129hml

Page No. \_\_\_\_\_

February 1992

98-6 Western to ascertain binding to GP160.

Run 12.5% SDS-PAGE.

Lane	Sample	
2	200ng gp160 (III B)	-- gp160 (III B) from S. Haem - Blot to NC - 1hr/120V - Block 30', RT, 5% Milk/PBS - Cut blot into 3 strips ① lanes 1-5 + lane 6 ② 1/2 lane 6 + lanes 7-8 ③ lanes 9-10
3	100ng gp160 "	
4	50ng gp160 "	
5	25ng gp160 "	
6	Rainbow Markers	
7	25ng gp160 (III B)	
8	50ng gp160 "	
9	100ng gp160 "	
10	200ng gp160 "	

Incubate strip #1 with 2/17/92 98-6 sup (43ng/ml) diluted 1:2 in PBS-T

Incubate strip #2 with 447D (20ng/ml) 1:1000 in PBS-T

Incubate strip #3 with Cos-Mock sup diluted 1:2 in PBS-T

RT 1hr.

-- wash blots 3 PBS-T, place in same container, and probe 1 GCM IgG-AP.

-- wash, detect ECL.

- ① 447D, even in long (10') exposure does not detect GP160 III B; 447D was selected against M strain and only reacts weakly with III B
- ② Mock control detects nothing.
- ③ 98-6 lights up GP160!! In 1' exposure can clearly see 200ng, 100ng, 50ng gp160 with very little background. In very long exposure (5' and 10') the rainbow markers are detected as well as a few other background bands.

February 1992

[RIP] 50 hour exposure:

- ① 98-6 does not precipitate F protein of RSV
- ② H1129<sup>wt</sup> and H1129<sup>Δ6</sup> do not precipitate RSV-F. Since the H1129<sup>Δ6</sup> chain does not (Lanes 50) this brings out the doubt about the H1129<sup>Δ6</sup> chains.

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To Page No. \_\_\_\_\_

Date

TITLE COS: H1129h+L; CA/H1129; H1308F: H66-71

ject No. 98  
Book No. 95

From Page No.     

## COS Transfection

H1129h+L-1B, C1, 7A

CH1129x, + H1129L-6A

H1308F: H66-71

27 February 1992

Seed 8 P100's @  $2 \cdot 10^6$  cells/plate/10ml, P58

28 February 1992

pp4 DNAs:

H1308F:66-71 (40x ca) 250ul h, 23ul d

H1129-1B: 19ul h, 36ul d

H1129-6A: 19ul h, 38ul d

H1129-7A: 19ul h, 53ul d

CH1129x/H1129L-6A: 23ul h, 38ul d

Spin, wash  $\bar{c}$  100% EtOH, decant in hood, dry.

Note Remaining infection steps by CTS up to DMSO shock.

DNA on cells at 12<sup>00</sup>; Shock cells @ 6<sup>00</sup> PM; Incubate with 12ml 10% NS medium.

2 March 1992

Harvest supernatant plates  $\bar{c}$  15ml 10% NS medium

## ELISA

H1129-1B:	450 ng/ml
H1129-6A:	77 ng/ml
H1129-7A:	32 ng/ml
CH1129-6A:	140 ng/ml
H1308F:66-71 #1:	450 ng/ml
H1308F:66-71 #2:	450 ng/ml

Values are very low! Either  $\bar{c}$  is a problem with the DNAs (had conc incorrect concentrations, not resuspended in TBS) there was a problem with the transfer

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Date 2/1/92

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Project No. 98  
Book No. 15

TITLE COS: H1129b-L; CH/H1129-CA; H1308F: H66-71

Page No. 1

3 March 1992

Set up 7 P100i @  $2 \cdot 10^6$  cells/plate/10ml, P60, for a repeat transfection including a positive control.

Precipitate DNAs:

H1129-1B: SJ81 (heavy chain) - 19  $\mu$ l  
light 1B - 36  $\mu$ l

H1129-6A: SJ81 (heavy chain) - 19  $\mu$ l  
light 6A - 38  $\mu$ l

H1129-7A: SJ81 (heavy chain) - 19  $\mu$ l  
light 7A - 53  $\mu$ l

CH1129x/H1129/6A: SJ75 (heavy chain chimera) - 23  $\mu$ l  
light 6A - 38  $\mu$ l

H1308F:H66-71: heavy - 25  $\mu$ l  
SJ61 (humanized light) - 11.6  $\mu$ l

H1308F:S71: SJ82 (humanized heavy S-71) - 3  $\mu$ l  
SJ61 (humanized light) - 11.6  $\mu$ l

Spin, decant, wash  $\approx$  100% EtOH, decant & dry in hood.  
to  $-70^\circ\text{C}$  O/N.

Dilute DNAs to be transfected to 100ng/ $\mu$ l in TE, run 200ng each on .8% agarose/TAE gel to be certain that the DNAs are intact and the concentrations are reasonably accurate.

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Date

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TITLE COS: H1129b-1; CH/H1129-6A; H1308F: H66-71; H1308F57 Book No. 95

Project No. 98

From Page No. 95

DNA's all look intact and all within  
5 fold of each other in concentration.

4 March 1992

Resuspend DNAs in 0.5ml TBS, add to 2.5ml DEAE-Dextran/  
DMEM and transfect 1-9100 each along with one mock p.

- DMEM cells at 90% con

- Shock cells @ 500 PM = 8 hrs; feed cells with 15ml

6 March 1992

Harvest supes from first transfection

ELISA (8.3.9.92):

H1308F:66-71 v1: 450 ng/ml  
H1308F:66-71 v2: 450 ng/ml  
H1129-1b: 450 ng/ml  
H1129-6A: 245 ng/ml  
H1129-7A: 925 ng/ml  
CH1129X/H1129-6A: 2.1 ng/ml

Note: H1308F:66-71 did not express  
at all in this transfection.  
H1129-1b did not express at  
all in this transfection.

Toss cultures.

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R-150/1

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3/1

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Project No. 98

Book No. 75

TITLE COS: H1129-h1, H1129-6A; H1308F:66-71

Form Page No. 1

7 March 1992

Harvest super from 3/4 transfection; feed plates &amp; 15ml medium.

## ELISA (3-9-92)

Mock: 450 ng/ml  
 H1308F:571: 1.4 µg/ml \*  
 H1308F:66-71: 450 ng/ml  
 H1129-1b: 450 ng/ml  
 H1129-6A: 60 ng/ml \*  
 H1129-7A: 450 ng/ml  
 CH1129/H1129-6A: 450 ng/ml

2 March 1992

Harvest super from 3/4 transfection (5 days); feed plates &amp; 15ml.

## ELISA

Mock: 450 ng/ml  
 H1308F:571: 2.17 µg/ml \*  
 H1308F:66-71: 450 ng/ml  
 H1129-1b: 450 ng/ml  
 H1129-6A: .99 µg/ml \* -- To SJ 3/18/92  
 H1129-7A: 68 ng/ml \*  
 CH1129/H1129-6A: 1.99 µg/ml \*

Still the H1308F:66-71 does not express, nor does the H1129-1b. The H1129-1b construct does have some errors which may explain the lack of expression. There is no apparent reason for the H1308F:66-71 not to express.

13 March 1992

T.O.S.S. transfection plates, cells are all pretty toasted!

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Invented by

T Page No.

Date

3/13/92

11  
FILE COS: H11296.2, CH/H1121 1; H1308F:66-71

Project No. 70  
Bc No. 95

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*[The main body of the form is a large grid with horizontal and vertical lines, crossed out by a diagonal line from the top-left to the bottom-right.]*

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Project No. 78

Book No. 95

TITLE \_\_\_\_\_

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#### PROTOCOL FOR ELISA-HU IgG QUANTITATION

##### A) BUFFER SOLUTIONS

- 1) Coating Buffer: Carbonate buffer, 0.1M, pH=9.6  
0.159 gm Sodium carbonate  
0.093 gm Sodium bicarbonate  
Adjust to pH 9.6, make up to 100 ml.
- 2) Washing Buffer: PBS, pH=7.4, with 0.1% Tween-20. +Dilexit
- 3) Blocking Buffer: PBS, pH=7.4, with 3% non-fat dry milk.

##### B) PROCEDURE

- 1) Add antigen: affinity purified Goat anti-human IgG  
(Fc fragment) (gamma chain specific) (Cappel cat. no. 34780) at 1 ug/well in 50-ul coating buffer. Incubate at room temperature (RT), in a humid chamber for 2 hours.
- 2) Flick solution of the wells.
- 3) Add 0.2 ml blocking buffer for 30 minutes.
- 4) Add 0.05 ml of Hu IgG standard solutions: Human IgG (whole molecule, chromatographically purified, Cappel cat. no. 5001-0080, lot no. 34428).  
Add 0.05 ml/well of the sample to be tested. Incubate for 1 hour, in a humid chamber at RT.
- 5) Wash 4X with washing buffer.
- 6) Add 0.05 ml of secondary antibody: Goat anti-human IgG  
peroxidase labeled (Cappel cat. no. 5005-5, lot no. 5005-5) diluted 1:1000 in PBS-1% Tween-20.  
RT 1hr.
- 7) Wash 4X with washing buffer.
- 8) Add 50 ul/well 4BTB peroxidase substrate (Kirkegaard & Perry, cat. no. 50-64-02, lot no. RH01). Incubate for 5 minutes, read at 405 nm.

#06010121  
Ref # 55261

\* 8601008  
Ref # 55261

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TITLE 13087 ELISA - 9-10-91

Prnt No. 98

Book No. 95

From Page No. 10

September 1991

Human IgG Quantitation #1

Follow procedure, P100

① Coat wells @ 1 µg/well x 100 wells = 100 µg/5ml DSP

① Coat Plate @ Goat Anti-Human IgG-Fc (from G. Bensa)

② Columns 1-4: 1 µg/well -- 32 µg/1.6 ml

③ Columns 5-8: 0.5 µg/well -- 16 µg/1.6 ml

④ Columns 9-12: 0.1 µg/well -- 3.2 µg/1.6 ml

Carbonate buffer

-- Incubate, RT, 2 Hrs: 10<sup>45</sup> - 12<sup>15</sup>

② empty wells, add 200 µl blocking soln; RT-30'

③ Dilute Stds: 1 mg/ml (1 µg/ml) stock -- dilute 1:10 = 100 ng/1 µl

320 ng/1 µl: 3.2 µl 100 ng/1 µl → 1 µl

-- Serially dilute 1:2 (350 µl → 350 µl) (500 µl → 500 µl) 10:

160 ng/1 µl

80 ng/1 µl

40 ng/1 µl

20 ng/1 µl

10 ng/1 µl

5 ng/1 µl

in PBS pH 7.4 / 1% Tween

④ Dilute Samples, 1:2 (200 µl → 200 µl) and 1:10 (40 µl + 360 µl)

⑤ Load Wells

	1	2	3	4
A	0.2	→	5366	916 1:2
B	5	→	5366	916 1:10
C	10	→	5366	916 1:2
D	20	→	5366	916 1:10
E	40	→	5366	916 1:2
F	80	→	5366	916 1:10
G	160	→	5366	915 1:2
H	320	→	5366	915 1:10

Repeat for  
1 µg/well, 0.5 µg/well  
and 0.1 µg/well of  
capture antibody

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Book No. 6

TITLE 1308FELIS 9-10-91

Page No. 1

### Results

calculate standard curve using 0, 5, 10, 20 ng/ml standards  
ETI calculator

- (1) punch in standard concentration (X-value)
- (2) Hit  $X \leftrightarrow Y$  key
- (3) punch in OD value (Y-value)
- (4) Hit  $\Sigma$  key

-- repeat for all values to be used in standard curve

- (5) punch in sample OD \* value
- (6) Hit  $X'$  key -- get value

Slope = .0098

Correlation coefficient = .99

} using 0 → 20 ng/ml

Sample	1 ng/ml Value	0.5 ng/ml	1 ng/ml
SJ66 9/6 1:2	* <del>37.4</del> 74.8 ng/ml	72.6 ng/ml	66.6 ng/ml
SJ66 9/6 1:10	66.1 ng/ml * 66.0 ng/ml	-- No results --	67.0 ng/ml
SJ60/61 9/6 1:2	* 30.6 ng/ml 33.8 ng/ml	30.4 ng/ml	43.2 ng/ml
SJ60/61 9/6 1:10	33.5 ng/ml * 32.0 ng/ml	32.0 ng/ml	28.0 ng/ml
SJ36NL 9/6 1:2	0	0	0
SJ36NL 9/6 1:10	0	0	0
SJ60/61 8/15 10% DS 1:2	-- off scale --	-- off scale --	-- off scale --
SJ60/61 8/15 10% DS 1:10	* 367 ng/ml	-- off scale --	397 ng/ml
Slope	* 0.01157	0.0116	0.0125
Correlation coefficient	* 0.9965	0.9953	0.9726

\*: 1 ng/ml recalculated, using 0 → 40 ng/ml  
Standards in standard curve

SJ66 9/6  $\bar{x}$  = 69.4 ng/ml  
SJ61/60 9/6  $\bar{x}$  = 33.8 ng/ml  
SJ61/60 8/15  $\bar{x}$  = 382.0 ng/ml

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Date

Invent d by

D. S. R. R. R.

Date

10/2/91

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11  
TITLE 1308F Purification: COS 8/9/91 Xfection Project No. 98  
book No. 95

From Page No. \_\_\_\_\_

1308F Purification: Protein G  
COS 8/9/91 Transfection: NS Supers

2 October 1991

Thaw COS/1308F supers at 4°C

3 October 1991

Prepare Amicon MAC-Protein G capsule (item #11039, 25mm dia)

- ① Wash 20ml PBS, ~1ml/min.
- ② Wash with 0.15M Glycine pH 2.3, 15ml, ~1ml/min.
- ③ Wash with 15ml PBS; eluate pH 7.5 at end.

Filtered 1308F 10% No Serum COS1 Supers, 8/9/91 Transfection, through  
2µ Cellulose Acetate filter.

Load filter disk

4°C (cold room), using Rainin Rabbit peristaltic pump at 799  
loaded 80ml sample @ 370.6µg/ml (CAB95-173);  
80ml loaded in 60min (~1.3ml/min)  
Total sample = 29.6µg

Wash disk with 20ml PBS, 1.3ml/min; save PBS wash.

Eluted disk with 0.15M Glycine, pH 2.3, 12ml. Collect 6-2ml  
aliquots into 15ml tubes containing 1ml 2M Tris Base, pH  
undisturbed; eluted using syringe.

Read 1280 of fractions:

1 - .275  
2 - .033  
3 - .020  
4 - .015  
5 - .015  
6 - .015

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Date

D-15R

11-10-91



Project No. 98Book No. 12TITLE 1308F Purification: Protein GPage No.     

Dialyze samples #1 → #4, BRL 3/4" diameter MWCO 12-14kd  
 prepared dialysis tubing, 3L PBS pH 7.4, 4°C.  
 4:50 PM → 6:50 PM

Change buffer; dialyze o/n.

October 1991

Remove samples to tubes: ~ 5ml each.

Read A280/A260:

Sample	A280	A260
1	.394	.436
2	.034	.039
3	.020	.025
4	.026	.029

Run 4-20% PAGE (reducing): 50µl sample + 10µl 4x loading buffer  
 -- 90°C 10'  
 -- load 50µl on Gel

Lane	Sample
1	Protein A Pre-load
2	Protein A flow thru
3	Protein A PBS wash
4	Protein A fraction 1
5	Rainbow Marker
6	Protein G Pre-load
7	Protein G flow thru
8	Protein G PBS Wash
9	Protein G fraction 1
10	Protein G fraction 2

Gel on LNB95-106.

Stain 1.5 hr, destain o/n, several changes.

TITLE 1308F Purification: IgG

Project No. 98

Book No. 95

From Page No. —

5 October 1991

Filter fraction #1 through  $0.2\mu$  filter into 15ml tubes to  $4^{\circ}\text{C}$

7 October 1991

IgG quantitation (by ELISA) LNB95-178:

Sample: 772 ng/ml

flow Thru: 603 ng/ml

PBS Wash: 0

Fraction 1: 0

Note: color development was poor in this assay. Also, applied sample 2 was the same as the applied sample 1. The Protein A column, which gave a value of only 240 ng/ml (LNB95-108).

8 October 1991

Concentrate fraction 1 using Centricon 30 concentrator

① load 2.5 ml

② Spin J120, 3000 RPM, 30',  $4^{\circ}\text{C}$

③ load another 2.0 ml; repeat step #2

④ final volume =  $\sim 900\mu\text{l}$  <sup>DSP</sup> 350  $\mu\text{l}$  = 13 fold concentrate

ELISA

Protein G Fraction 1: 0 ng/ml

Protein G Fraction 1 concentrate: 34 ng/ml

The Ig seen on the gel is mostly Bovine Ig, which will adsorb protein G (FBS has  $\sim 920\text{ ng/ml}$  Ig, = 920  $\mu\text{g/ml}$  in 10% FBS mtd. so there is >1000 fold more bovine than 1308F Ig in the sample.

Only 13.5  $\mu\text{g}$  (of a total of 61.8  $\mu\text{g}$ ) 1308F bound to the column, dialysis and concentration, only 11.9 ng remained. >1000 fold loss. The possibility that the 1308F aggregated and was lost by filtering after dialysis can not be checked since there is no pre-dialysis sample. Keep column flow thru, loss res. T Pag N

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Date

10/8/91

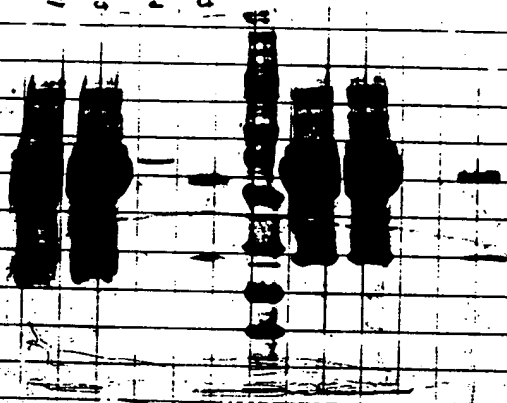
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TITLE 1308F Peritration Protein G

m Page No.     

Protein A			Protein G			
Substrate	Enzyme	Condition	Control	Substrate	Enzyme	Condition
1	2	3	4	5	6	7



L2895-104,107  
10-4-91

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Invented by

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TITLE 1308F Purification & Transfection; Protein A

Project No. 98

Book No. 95

From Page No. 1308F Purification: Protein A

COS 8.9.91 Transfection, NS Pooled Suprs

4 October 1991

Prepared Amicon MAC-Protein A Capsule (#11035)

- ① Wash with 25ml PBS, syringe
- ② Wash with 20ml 0.15M Glycine pH 2.3, syringe
- ③ Wash with 25ml PBS, syringe. Final eluate = pH 7.5

Loaded 80ml sample (LNB 95-103) onto capsule by peristaltic pump, ~1.3ml/min, in cold room.  
-- Total sample = 29.6  $\mu$ g

Wash disk with 20ml PBS, save wash;  $A_{280}$  of last ml = .03

Elute with 12ml 0.15M Glycine pH 2.3; collect 2ml fract in tubes containing 1ml 2M Tris Base, pH unadjusted.

$A_{280}$  of samples:

1-	.191
2-	.022
3-	.013
4-	.014
5-	.016
6-	.014

Dialyze fractions #1, 2 against PBS at 4°C (3L):

- ① 9<sup>40</sup> → 12<sup>10</sup>, change buffer
- ② 4°C O/N

Run Gel on. Preload, flow thru, PBS wash, fraction #1 @  
-- LNB 95-104 for Gel info, Gel LNB 95-106.

5 October 1991

Remove samples to 15ml tubes; 4°C O/N

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Date

Invented by

Date

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Book No. 5

TITLE 1308F Purification 1991 Transfection; Protein A

Page No. \_\_\_\_\_

7 October 1991

IgG quantitation (by ELISA) LNB95-178:

Sample: 240 ng/ml

Flow Thru: 35 ng/ml

PBS Wash: 0

Fraction 1: 443 ng/ml

Note: color development was slow and low. The applied sample was the same as for the Protein G applied sample (LNB95-105) which had a value of 772 ng/ml.

8 October 1991

Concentrate Fraction 1 using Centricon 30 concentrator:

① apply 2.3 ml to concentrator

② spin 30', 3000 RPM, 4°C JA20

③ apply 2 ml more, repeat step #2

④ final volume = 420  $\mu$ l = 10 fold concentration

ELISA

Protein A fraction 1: 58.3 ng/ml

Protein A fraction 1 concentrated: 415.7 ng/ml

Total sample bound to protein A = 16.4 ng  $([Sample - Flow thru] \times volume)$

Total sample recovered = 2 ng  $(443 ng/ml \times 4.5 ml) = 12\% recovery$

The concentrated sample was 7 fold more concentrated than the dialyzed sample, even though the volume was reduced 10 fold, indicating that some sample was lost to the concentrator. The dialyzed sample was 7 fold ~~more~~ less concentrated than the initial column fraction indicating that some of the sample was lost in dialysis -- adsorbed to the membrane or leakage.

Save concentrated fraction 1.

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Ingressed & Understood by me,

Date

Invented by

D. C. P. / L. A. /

Date

10/8/91

Project No. 98

Book No. 5

TITLE 130RF 8.9.91 Transfection: Protein A #2

m Page No.     

130RF 8.9.91 Cos Transfection  
Protein A Purification #2.

October 1991

PBS wash protein d disk, .15M pH 2.3 glycine wash, PBS wash.

Load Protein A (LUB 95-107) and Protein G (LUB 95-103) column.

flow-Thru samples onto column -- 6ml/ml

Pass through column 3 times.

Wash column with 25ml PBS

Elute column with 12ml .15M Glycine pH 2.3 -- collect 3-2ml fractions into 1ml 2M Tris base.

Read A280 and A260 of fractions:

	A280	A260
1	.209	.171
2	.042	.050
3	.040	.049

-- Toss fractions 2 and 3.

Dialyze fraction 1 against PBS -- 2L @ 5<sup>30</sup> PM.

8<sup>30</sup> PM -- change buffer; 4°C O/N.

3 October 1991

Concentrate fraction 1 using Amicon centricon 30 concentrator

① load 2.3ml into concentrator

② Spin 3000RPM/30'/4°C J120

③ load additional 2ml, repeat step 2

④ final volume = 420ul = 10 fold concentration

ELISA

Protein A fraction 1: 176ng/ml

Protein A fraction 1 concentrated: 425ng/ml

Witnessed & Understood by me,

Date

Invented by

J. S. R. Linn

Date

10/19/91

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TITLE 1308F 8.9.91 Transfection: Protein A #2

Project No. 198  
book No. 95

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Again, the sample was concentrated 10 fold, but the 1308F4 concentrated only 2.4 fold -- must be adhering to the conc

Save Protein A2 fraction 1 concentrate.

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Invented by

TS R/au

Date

10/10/91

To Page 1

TITLE 1308F Purification: Human IgG Agarose

Pact No. 98  
Book No. 95

10-9-91

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10 October 1991

ELISA:

1308F NS Pool	-	219 ng/ml : 5ml aliquot
1308F NS Pool filtered	-	268 ng/ml : 5ml aliquot
Column Flow Thru	=	1 ng/ml
PBS Column Wash	=	1 ng/ml - TOSS
PBS Column Wash #2	=	1 ng/ml
Fraction #1	=	60 ng/ml : 6ml
Fraction #2	=	0 ng/ml
Fraction #3	=	0 ng/ml - TOSS
DEAE Sepharose Strip	=	0 ng/ml

All the 1308F bound to the column (268 ng/ml on, 1 ng/ml flow to none eluted in the wash, and very little eluted in the elution step or in the strip steps -- or all is being lost the dialysis step.

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Witnessed & Understood by me,

Date

Invented by

Date



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TITLE 1308F Purification: Human IgG-Agarose (Tony's)

Project No. 98

Book No. 95

10-1991

From Page No. 1308F Purification

Human IgG-Agarose Column (Tony)

18 October 1991

Will Purify 1308F from 8.9.91 COS Transfection, NS Prod.

Pour ~1ml bed volume Anti-Human IgG-Agarose (from T. Ruiz) column with PBS until UV trace is linear.

Load 200ml conditioned medium sample onto column by gravity flow (~2ml/min flow).

PBS wash until UV trace is linear.

Elute with 0.5M  $\text{NH}_4\text{OAc}$  pH 3.0 (from Tony Ruiz); collect 2 fractions (~2.5ml each) and neutralize with .5ml 1M Tris.

Remove 100ul aliquot from each sample -- store at 40C.

Dialyze fraction #2 (~2.2ml) and 1/2 of fraction #1 (~2ul) ago 2L PBS o/v.

-- other 1/2 of fraction #1: buffer exchange on centricon microconcentrator:

- ① apply 2ml sample to Centricon 30
- ② Spin 25min, 3000RPM, J120, 40C; volume = ~1.5ml
- ③ add 1.5ml PBS, pipette up and down to wash
- ④ spin 25min, 3000RPM, J120, 40C; volume = ~1.8ml
- ⑤ add 1.5ml PBS, wash.
- ⑥ Spin 30min, 3000RPM, J120, 40C. Final volume = ~.
- ⑦ Remove concentrated sample to new tube, 40C. 6 concentrator membrane with .3ml PBS, save at 1

19 October 1991

Change dialysis buffer.

21 October 1991

Remove dialysates to new tubes. Filter sterilize #1 & dialysates, and #1 concentrated with .2um low protein binding fi

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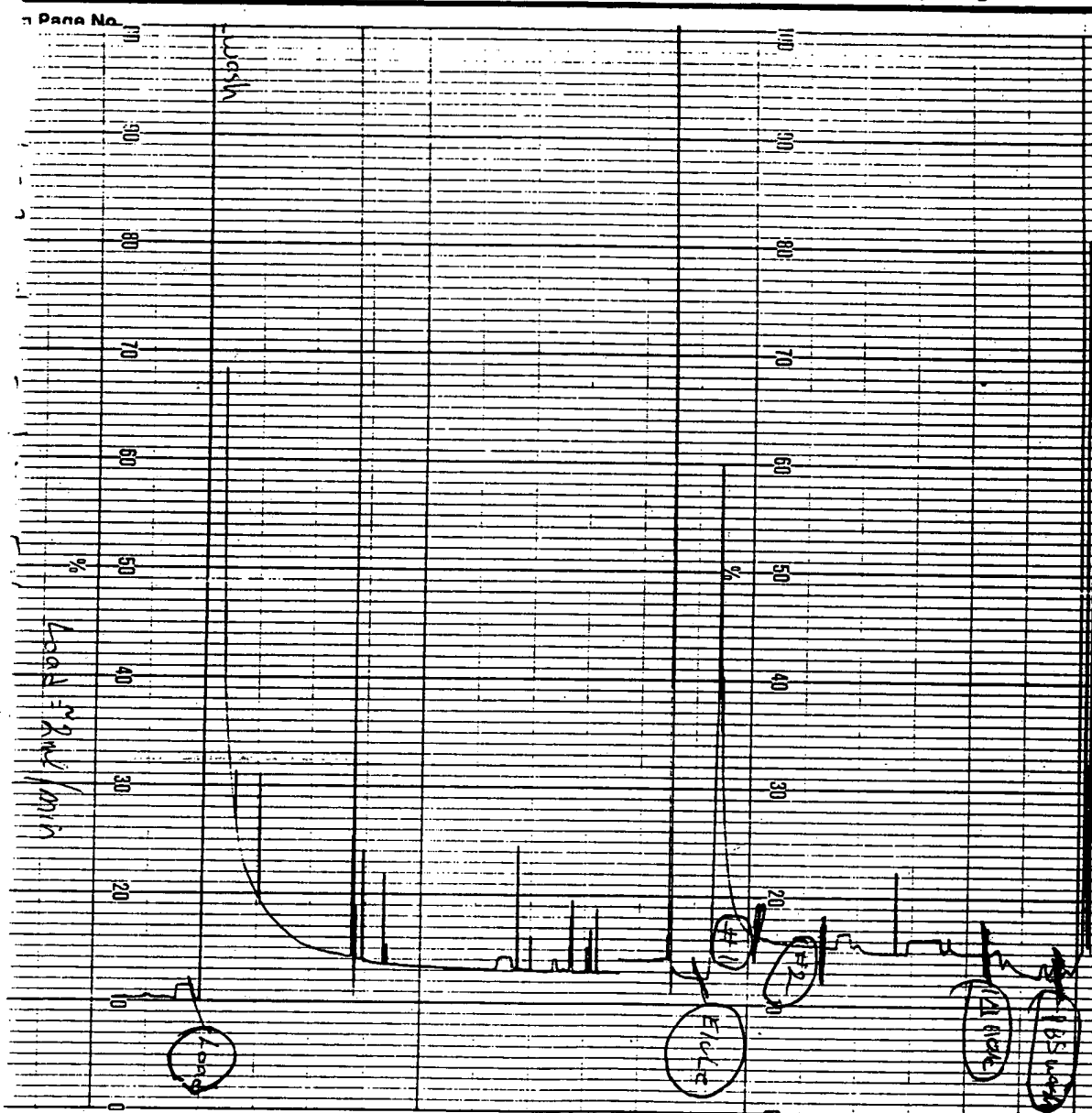
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Project No. 98  
Book No. 5

**Book No.** 5

TITLE 1308F Purification of Human IgG-Agarose (to



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Invent d by

Invent d by D. J. S. R.

Date \_\_\_\_\_

10/10/10

TITLE 1308F Purification:  $\alpha$  man IgG-Agarose (Tony's).

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22 October 1991

ELISA: (10/18, 10/21, 10/22):

Sample	Volume	[1308F]	Total 1308F
Conc. Start Medium	200ml	202 ng/ml	40.4 $\mu$ g
Flow Thru	200ml	14.7 ng/ml	2.9 $\mu$ g
Fraction #1	4.2 ml	3.7 ng/ml	15.5 $\mu$ g
Fraction #2	2 ml		
#1 dialyzed	2.4 ml	2.8 ng/ml	6.7 $\mu$ g
#1 dial. and filtered	2.4 ml	3.2 ng/ml	7.7 $\mu$ g *
#2 dialyzed	2.4 ml	.48 ng/ml	1.2 $\mu$ g
#2 dial. and filtered	2.4 ml	.82 ng/ml	2.0 $\mu$ g *
#1 concentration	.75 ml	8.1 ng/ml	6.1 $\mu$ g
#1 concentration and filtered	.75 ml	8.8 ng/ml	6.6 $\mu$ g *

\* Total recovery = 16.  
 40% recovery

Using Tony's column resin and reagents, I can purify 1308F but with a poor recovery.

23 October 1991

Run 2 gels on purified material: 12.5% PAGE.

For Western

Lane
1 - 500ng Murine 1308F
2 - 50ng Murine 1308F
3 - Rainbow Markers
4 - 100ng Purified 1308F
5 - 50ng Purified 1308F
6 - Blank
7 - 10ng Human IgG1, k
8 - 25ng "
9 - 50ng "
10 - 100ng "

For Coomassie

Lane
1 - 500ng Murine 1308F
2 - 50ng " "
3 - Rainbow Markers
4 - 100ng Purified 1308F
5 - 50ng " "
6 - 10ng Human IgG1, k
7 - 25ng " "
8 - 50ng " "
9 - 100ng " "
10 - Blank

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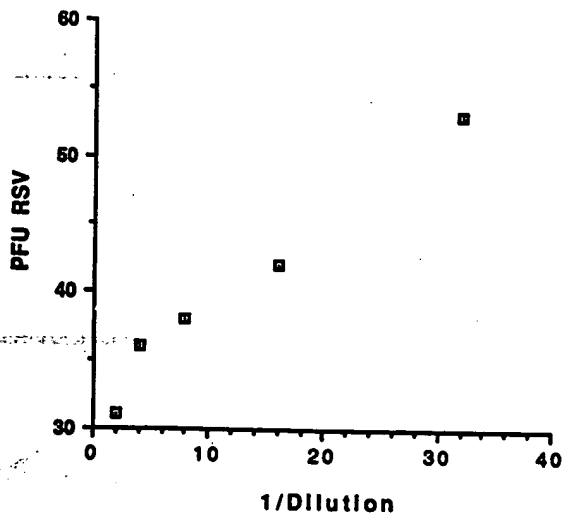
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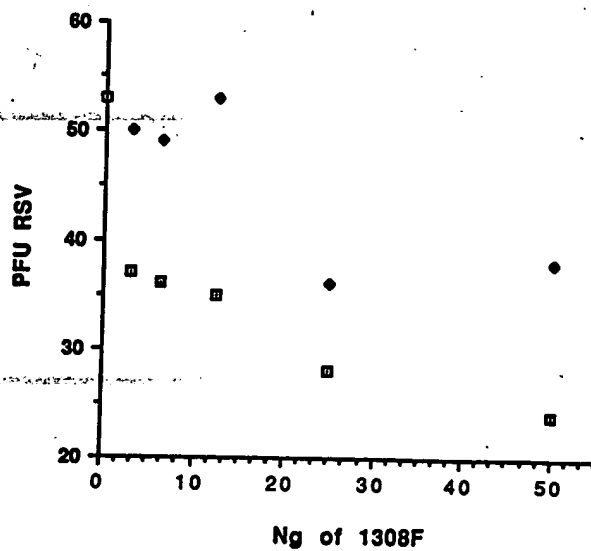
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10/28/91

### RSV Neutralization



■ Cos Super



■ Hu1308F

● Mu1308F

1308F Purification

$\alpha$ Human IgG: Agarose Comparison

22 October 1991

Pool 9/13/91 cos transfection FBS supernatants (Blue):

SJ60/61

SJ66

9/13  $\rightarrow$  9/16

9/13  $\rightarrow$  9/16

9/16  $\rightarrow$  9/20

9/16  $\rightarrow$  9/20

9/23  $\rightarrow$  9/27

9/23  $\rightarrow$  9/27

-- add  $\frac{1}{2}$  of 8.9.91 10% FBS pool to each of the above pools  
(total volume =  $\sim$ 250ml each).

Wash 2  $\sim$ 1ml bed volume columns ( $\alpha$ Human IgG: Agarose, one my lot  
other Tony's lot) with PBS.

Load columns simultaneously using peristaltic pump,  $\sim$ 5ml/min.

① equal volume through each column.

② pass flow thru over columns, individually a second time.

Wash columns  $\approx$  20ml PBS.

Elute columns with Tony's 5M  $\text{NH}_4\text{OAc}$ , pH 3

-- neutralize fractions to pH 7.0 with 2M Tris Base.

Dialyze samples (2 fractions each) against PBS o/n.

23 October 1991

Change dialysis buffer -- 2L @ 7:30 AM.

3:30 PM - remove samples to 15ml tubes; 4°C.

DSP Column, Fraction #1 - 5.5ml

" Fraction #2 - 6.5ml

Tony's Column, fraction #1 - 4.5ml

" fraction #2 - 2.3ml

} remove 0.2ml each to  
Sarstedt tubes; store @ -2

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TITLE 1308F Purification: Dose-Response Comparison

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25 October 1991

ELISA:

	DSP Column	Tony's Column
Column Flow Thru	63.5 ng/ml * (7.9 $\mu$ g)	41.0 ng/ml * (5.1 $\mu$ g)
Fraction #1	1.08 ng/ml (5.9 $\mu$ g)	3.09 ng/ml (13.9 $\mu$ g)
Fraction #1, dialyzed	1.19 ng/ml * (6.5 $\mu$ g)	3.18 ng/ml * (14.3 $\mu$ g)
Fraction #2	3.54 ng/ml (23 $\mu$ g)	0 (0)
Fraction #2, dialyzed	2.91 ng/ml * (18.9 $\mu$ g)	609 ng/ml * (1.4 $\mu$ g)
	* Total: 33.3 $\mu$ g	* Total: 20.8 $\mu$ g

running (loading) the column at such a high flow rate decreased the efficiency of binding of the 1308F

8 October 1991

Combine DSP fractions 1 and 2 with Tony fractions 1 and 2:  
 $\sim 40 \mu\text{g} / 18 \text{ ml} = 2.2 \mu\text{g/ml}$

Save 3ml in 15ml tube, pass remainder over 0.3ml bed volume Protein A-Agarose column.

- ① Pass flow thru over column a second time
- ② Wash column  $\pm$  4.5ml PBS, save wash (15 bed vol.)
- ③ Elute column with 0.15M Glycine pH 2.3  
 $\therefore$  collect 125ml fractions into 125ml 2M Tris Base  
 $\therefore$  Dialyze against PBS in Microdialyzer, 35umol, 2.5 hr

9 October 1991

ELISA

Sample	[1308F]	Volume	Total 1308F	%
Load	2.2 $\mu\text{g/ml}$ <del>60.5 ng/ml</del>	15ml	33.3 $\mu\text{g}$	100%
Flow Thru	60.5 ng/ml	15ml	0.91 $\mu\text{g}$	2.7%
PBS Wash	12 ng/ml	4ml	0.048 $\mu\text{g}$	0.1%
Fraction 1	20.1 ng/ml	0.45ml	9.05 $\mu\text{g}$	27.2%
Fraction 2	0.98 $\mu\text{g/ml}$	0.35ml	.34 $\mu\text{g}$	1%
Fraction 3	0		0	—
Fraction 4	0		0	—
Fraction 5	0		0	—
Fraction 6	0		0	—
			0	—

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D. F. S. P.

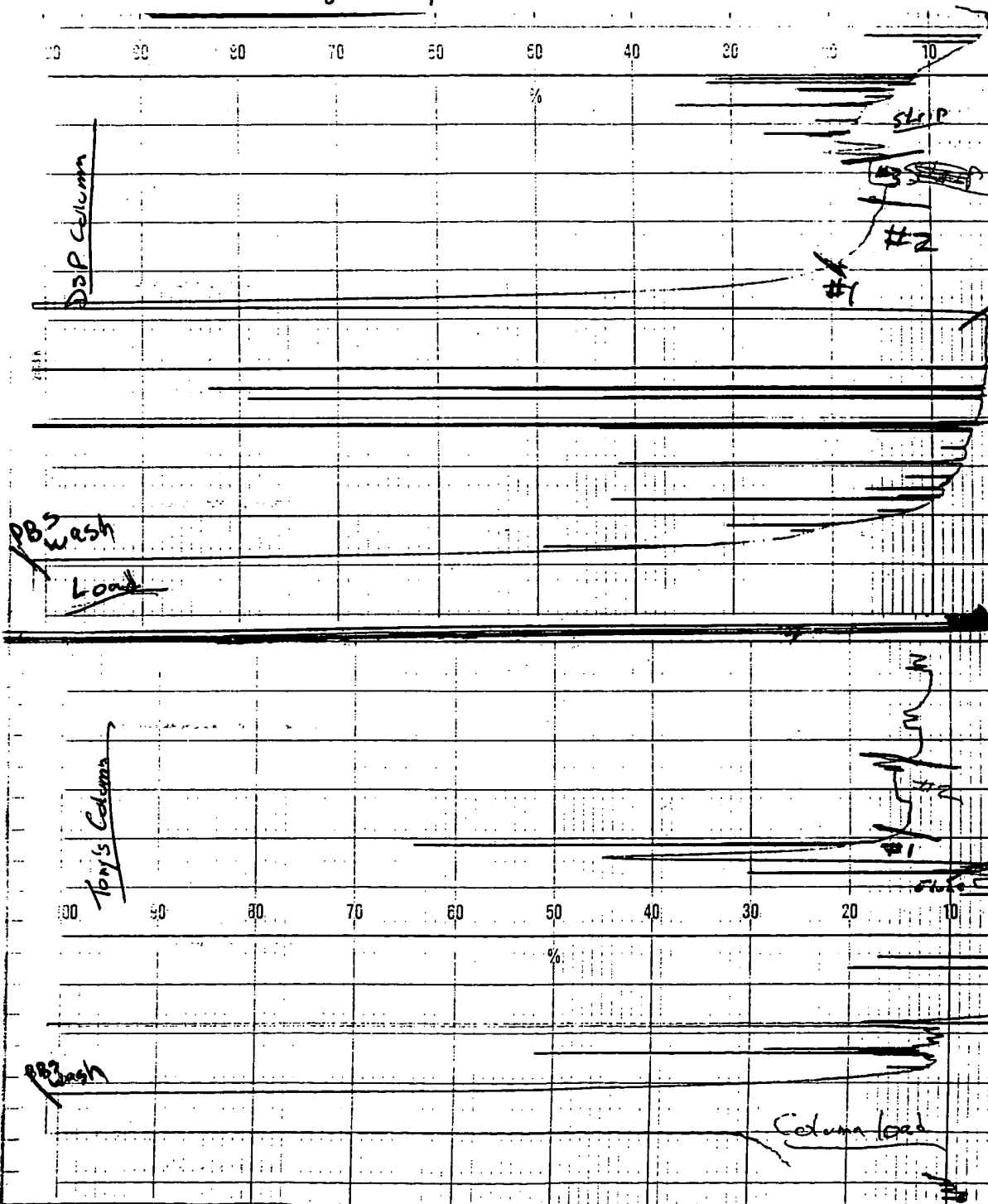
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TITLE 1308F Purification:  $\alpha$  Human Agarose Comparison

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Project No. 8Book No. 42TITLE 1308F Purification of Human  $\gamma$  ProteinPage No.     

of the 33  $\mu$ g of 1308F sample loaded onto the column,  
only 10.3  $\mu$ g (31%) can be accounted for. This loss is  
too great!

1 October 1991

Run 2 - 12% SDS/PAGE:

## #1 - Coomassie Stain

Lane	Sample
3	Rainbow Standards
4	500 $\mu$ g Murine 1308F: 6 $\lambda$ 100 $\mu$ g/ $\lambda$ + 34 $\lambda$ 1x dye; load 33 $\mu$ l
5	Blank
6	500 $\mu$ g Hu 1308F Protein fraction #1: 30 $\lambda$ 20.1 $\mu$ g/ $\lambda$ + 10 $\lambda$ 4x dye;
7	Blank
8	500 $\mu$ g Hu IgG1, k: 6 $\lambda$ 100 $\mu$ g/ $\lambda$ + 34 $\lambda$ 1x dye; load 33 $\mu$ l.

## #2 - Western Blot

Lane	Sample
1	Rainbow Standards
2	Hu 1308F, 100 $\mu$ g - $\gamma$ Human column (Tony) fraction #1 dialyzed; 63 $\lambda$ + 20 $\lambda$ 4x
3	Blank
4	Hu 1308F, 100 $\mu$ g - $\alpha$ Human Protein fraction #1: 10 $\lambda$ + 50 $\lambda$ 1x dye; load 30
5	Blank
6	200 $\mu$ g Hu-IgG1, k
7	100 $\mu$ g "
8	50 $\mu$ g "
9	25 $\mu$ g "
10	Blank "

Stain one gel, blot #2 to NC. Block blot o/N in 5% Milk/PB

November 1991

Wash blot, probe  $\epsilon$  G $\alpha$ H IgG-HRP. Wash ECL D $\alpha$  Erck.

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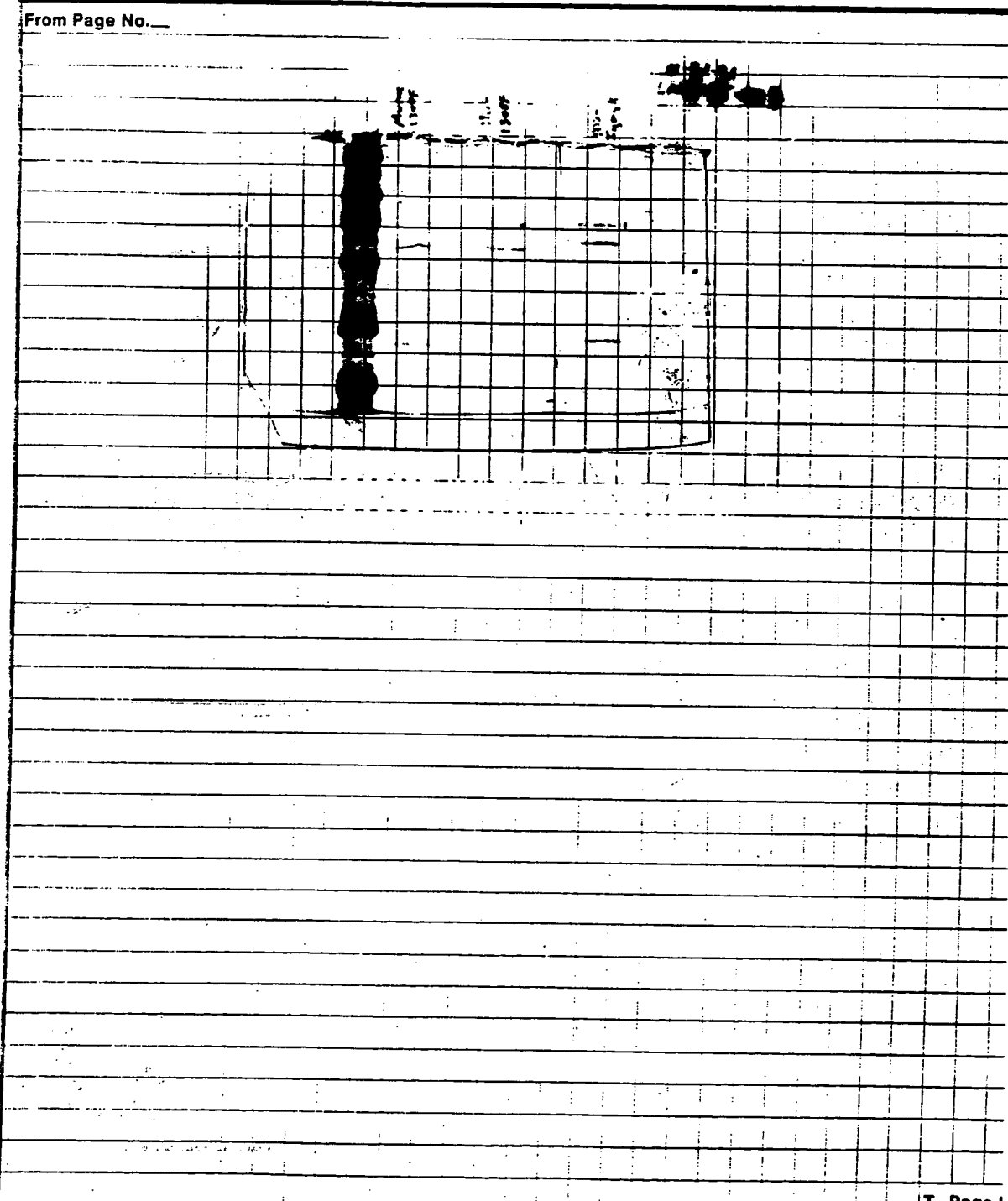


TITLE BC8F Purification: Human / Pictin A

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FILE 1308F: SJ66/MS Pool HU Purification

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on Page No. \_\_\_\_\_

1308F: SJ66/MS Pool (COS)

$\alpha$  Human IgG Purification

6 November 1991

Pool all COS-MS Pool - SJ66 supers remaining:

Teal 9/23  $\rightarrow$  9/27 120 ml

Teal 9/27  $\rightarrow$  9/30 110 ml

Teal 9/30  $\rightarrow$  10/4 115 ml (144 ng/ml)

Teal 10/4  $\rightarrow$  10/8 115 ml (172 ng/ml)

also combine with above COS-FBS - SJ66 supers:

Blue 9/20  $\rightarrow$  9/23 80 ml (149 ng/ml)

Teal 9/20  $\rightarrow$  9/23 40 ml

580 ml Total

Load  $\sim$  1.5 ml bed volume  $\alpha$  Human IgG-agarose column  $\bar{c}$  Pooled supers at 3.5 ml/minute

Wash Column  $\bar{c}$  PBS -- collect first 5 ml wash, continue to wash until UV baseline is stable ( $\sim$  40 ml total).

Elute column with 0.5M  $\text{NH}_4\text{OAc}$  pH 3.0: 450 ml  $\text{H}_2\text{O}$

14.5 ml Glacial Acetic Acid

$\text{NH}_4\text{OH}$  to pH 3.0

-- bring up to 500 ml

-- collect  $\sim$  3 ml fractions, neutralize to pH 7.5 with 2M Tris base

Strip column with 1M Acetic Acid -- collect eluate; elute  $\bar{c}$  0.5M  $\text{NH}_4\text{OAc}$ , collect  $\bar{c}$  1M strip. Adjust pH  $\bar{c}$  2M Tris Base.

Dialyze 3 fractions and column strip against 3L PBS o/v

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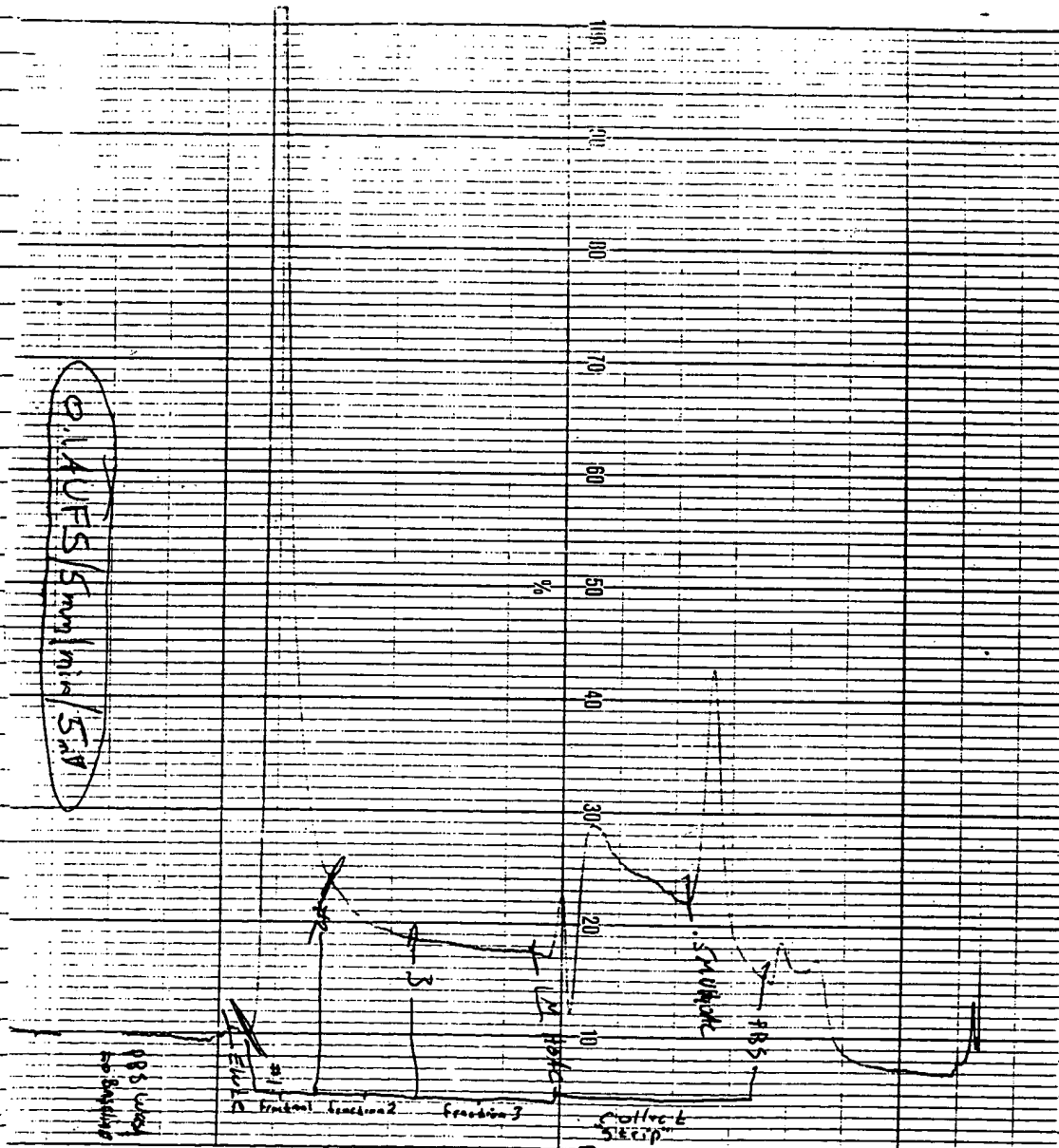
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TITLE 1308F: SJ66 Co. pool & Human Purification

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TITLE 1308F: SJ66C05 Pool in Human Perturbation

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7 November 1991

Change dialysis buffer, dialyze <sup>DSO</sup> 5HES. O/N.

8 November 1991

2mm filter samples, to 4°C:

Fraction 1: 4.2ml

Fraction 2: 4.6ml 5ml

Fraction 3: 3.6.7ml

Column Strip: 11.6ml

ELISA

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Date

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TITLE 1308F Purification SJ66 FuciaProject No. .98Book No. 95From Page No.     1308F Purificationfrom SJ66 Fucia  $10^4$  MTX lines 1, 2, 3

Want to purify a large quantity of hu1308F from ~~cells~~ <sup>DSP</sup> cells to use for ①  $K_0$  determination, ② in vitro virus neutralization, ③ in vivo virus neutralization in cotton rats.

10 January 1992

Count flasks of SJ66 Fucia  $10^4$  MTX #1, #2, and #3:

$$\#1 - 1.02 \times 10^6 \text{ cells/ml} \times 140 \text{ ml} = 1.43 \times 10^8 \text{ cells}$$

$$\#2 - 1.31 \times 10^6 \text{ cells/ml} \times 140 \text{ ml} = 1.83 \times 10^8 \text{ cells}$$

$$\#3 - 8.9 \times 10^5 \text{ cells/ml} \times 150 \text{ ml} = 1.34 \times 10^8 \text{ cells}$$

-- Note: these cells are clumped and the large clumps are not represented in these cell counts.  $\therefore$  these cell counts are underestimates of total viable cell #!!

SJ66 Fucia #1: seed entire volume into 500ml spinner with an additional 500ml  $\alpha$ -10% FBS/L-Glu/ $10^4$  MTX; Volume  $\therefore$  have  $\geq 2.23 \times 10^5$  cells/ml  
P13-3.

SJ66 Fucia #2: seed entire volume into 1l spinner with an additional 1l medium; Volume = 1140ml  $\therefore$  have  $\geq 1.61 \times 10^5$  cells/ml  
= P13-3

SJ66 Fucia #3: seed entire volume into 500ml spinner with an additional 500ml medium; Volume = 650ml  $\therefore$  have  $\geq 2.06 \times 10^5$  cells/ml  
= P13-3.

-- Cells seeded at 5<sup>00</sup> PM.

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TITLE 1308F Purification: SJ66 Fucia

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3 January 1992

Cell counts, 7<sup>30</sup> AM (= ~62 hrs) and samples for ELISA:

SJ66 Fucia #1 :  $5.2 \times 10^5$  cells/ml, 68% viable

[IgG 1, k]  
8.1  $\mu$ g/ml

SJ66 Fucia #2 :  $6.7 \times 10^5$  cells/ml, 94% viable

9.1  $\mu$ g/ml

SJ66 Fucia #3 :  $4.9 \times 10^5$  cells/ml, 96% viable

14.2  $\mu$ g/ml

4 January 1992

cell counts (9<sup>00</sup> AM) and samples for ELISA = ~88 hrs

SJ66 Fucia #1  $1.1 \times 10^5$ /ml, 16% viable

[IgG 1, k]  
11.4  $\mu$ g/ml

SJ66 Fucia #2  $3.9 \times 10^5$ /ml, 53% viable

11.2  $\mu$ g/ml

SJ66 Fucia #3  $2.5 \times 10^5$ /ml, 61% viable

16.6  $\mu$ g/ml

3<sup>00</sup> PM (94 hrs): Spin cells 1200 RPM, 3', Beckman J6-B.

.22  $\mu$  (cat) filter culture medium, store at 4°C o/n

15 January 1992

- Pour ~8 ml bed volume Goat  $\alpha$  Human IgG-Agarose (Sigma #A354, lot 059F-4883).

- Wash  $\pm$  2 bed volumes 4% Acetic Acid

- Wash  $\pm$  60 ml PBS, eluate pH 7.4 at end

- Load SJ66 Fucia #3  $1/10 \rightarrow 1/4$  (16.6  $\mu$ g/ml,  $\times 640$  ml) using Blue/Yellow pump tubing, pump set at 790 = 4 ml/min.

- Wash  $\pm$  PBS until UV trace returned to baseline

- Elute  $\pm$  .5M NH<sub>4</sub>OH pH 3.0, collect 4 ~6 ml fractions, neutralize to pH 7.5-8.0 with 2M Tris base

- Wash column  $\pm$  PBS

- Strip column  $\pm$  40% Acetic Acid; collect & neutralize eluate

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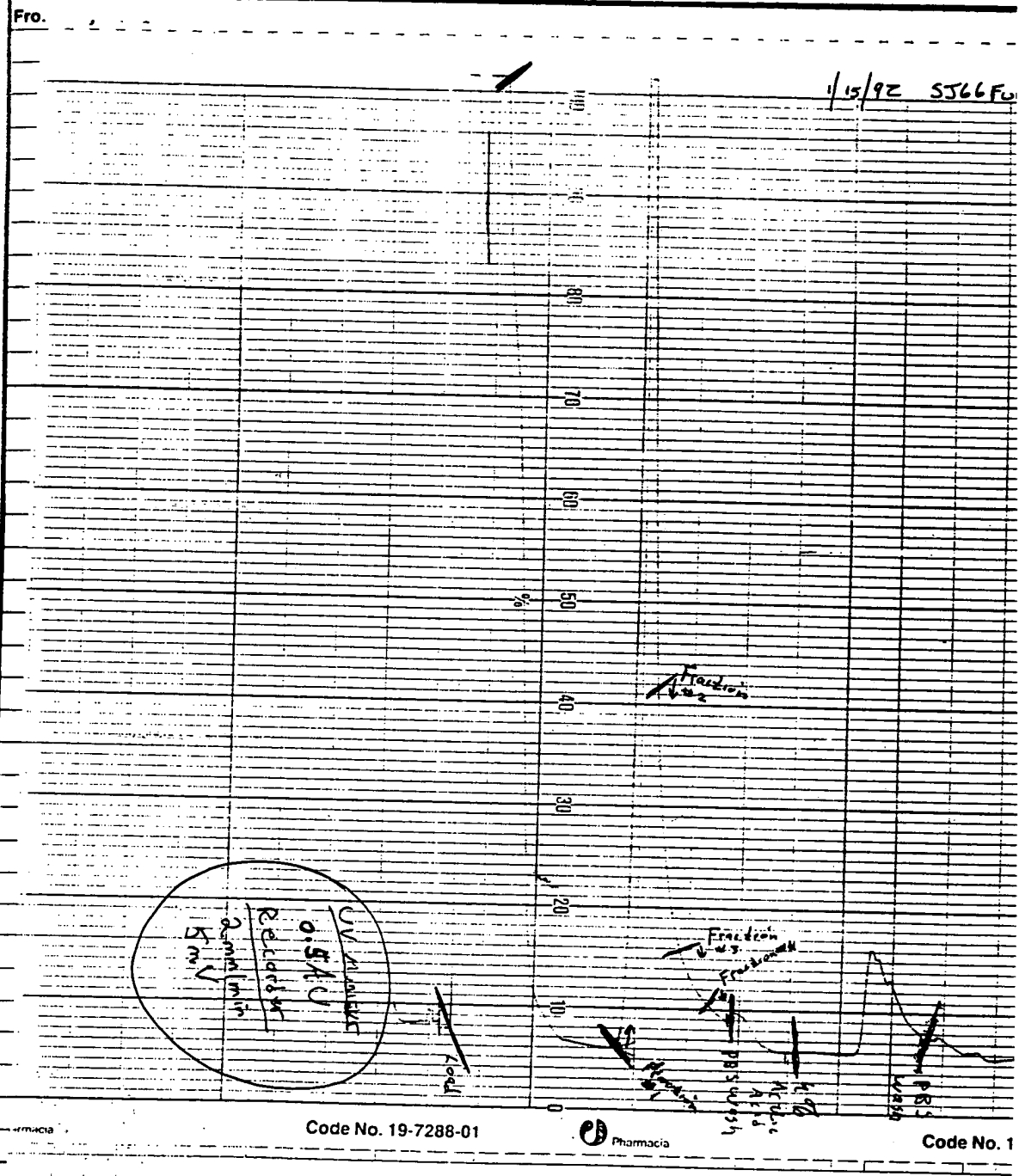
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TITLE 1308F Purification: 566 Fucia #3

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TITLE 1308F Purification: SJ66 Fura #3

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- Dialyze 4 fractions and 4% H<sub>2</sub>O<sub>2</sub> strip against 4L PBS, 4°C, 3 1/2 h

- Change buffer, dialyze o/n.

January 1992

Assay samples

① Column Sample  $13.77 \text{ mg/ml} \times 640 \text{ ml} = 8.81 \text{ mg}$

② Flow Thru #1  $0.55 \text{ mg/ml} \times 400 \text{ ml} = 0.22 \text{ mg}$

③ Flow Thru #2  $9.01 \text{ mg/ml} \times 240 \text{ ml} = 2.16 \text{ mg}$

④ fraction #1  $370 \text{ mg/ml} \times 6.5 \text{ ml} = 2.4 \text{ mg}$

⑤ fraction #1 dialyzed  $311 \text{ mg/ml} \times 7.7 \text{ ml} = 2.39 \text{ mg}$   $OD_{280} = .445 = 318 \text{ mg}$

⑥ fraction #2  $89.7 \text{ mg/ml} \times 6.5 \text{ ml} = 0.58 \text{ mg}$

⑦ fraction #2 dialyzed  $83.0 \text{ mg/ml} \times 8.4 \text{ ml} = 0.7 \text{ mg}$   $OD_{280} = .114 = 81 \text{ mg}$

⑧ fraction #3  $32.4 \text{ mg/ml} \times 7 \text{ ml} = 0.23 \text{ mg}$

⑨ fraction #3 dialyzed  $27.3 \text{ mg/ml} \times 9.5 \text{ ml} = 0.26 \text{ mg}$   $OD_{280} = .039 = 27.8$

⑩ fraction #4  $8.4 \text{ mg/ml} \times 4.5 \text{ ml} = 0.038 \text{ mg}$

⑪ fraction #4 dialyzed  $7.0 \text{ mg/ml} \times 6.5 \text{ ml} = 0.046 \text{ mg}$   $OD_{280} = .022 = 15.5$

⑫ 4% H<sub>2</sub>O<sub>2</sub> strip  $21.7 \text{ mg/ml} \times 7 \text{ ml} = 0.152 \text{ mg}$

⑬ 4% H<sub>2</sub>O<sub>2</sub> strip dialyzed  $21.3 \text{ mg/ml} \times 10.2 \text{ ml} = 0.217 \text{ mg}$  } Loss

2) Of the 8.81 mg total h1308F in the starting sample, only 5.99 mg (68%) can be accounted for in the flow thru and the fractions. Some may have been lost in the PBS wash, but 32% seems a bit high.

3) 3.4 mg (39%) of the h1308F can be accounted for in the fractions collected. The trailing nature of the fractions may be due to too high of a flow rate during elution (4 ml/min). I will decrease the flow rate during the elution in the future.



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TITLE 1308F Purification SJ66 Fucia #3

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③ 4% of the h1308F came through in the 1<sup>st</sup> collected flow thru (the first loaded onto the column) and 65% came through in the 2<sup>nd</sup> collected flow (the last 240ml loaded). Apparently the column capacity was exceeded so late in the loading and all of the h1308F began to flow thru. I will more G4H IgG-agarose and pour a larger bed volume column in the future.

#### SJ66 Fucia #3 Flow Thru Purification

Since there is 2.38 mg (27%) of the starting h1308F in the flow thru will attempt to purify it.

-- Rinse the G4H IgG-agarose column  $\bar{c}$  PBS, strip  $\bar{c}$  4% H<sub>2</sub>OAc, and rinse  $\bar{c}$  with PBS.

-- Load the 1<sup>st</sup> + 2<sup>nd</sup> column Flow thru from 1/15/92 onto the column  $\bar{c}$  2.5ml (Blue-Yellow pump tube, setting 500): start at 5:15 PM, stop at 9:20

-- Wash the column  $\bar{c}$  60ml PBS, UV trace to baseline.

-- Elute slowly (setting 350)  $\bar{c}$  0.5M NH<sub>4</sub>OAc pH 3.0, spike elution buffer  $\bar{c}$  ~25% 4% H<sub>2</sub>OAc after the second fraction (i.e. for fraction 3). Collect fractions.

-- Strip column  $\bar{c}$  4% H<sub>2</sub>OAc, wash  $\bar{c}$  PBS, store in PBS/1% azide.

-- Dialyze 3 fractions (fraction 1 = ~~11.4ml~~<sup>10ml</sup>, fraction 2 = ~4ml, fraction 3 = ~9.5ml) against 2L PBS o/n, 4°C.

17 January 1992

Change dialysis buffer, dialyze 3hrs, 4°C.

Remove samples to 15ml polypropylene tubes:

Fraction 1 = 11.4ml

Fraction 2 = 8.3ml

Fraction 3 = 11.7ml

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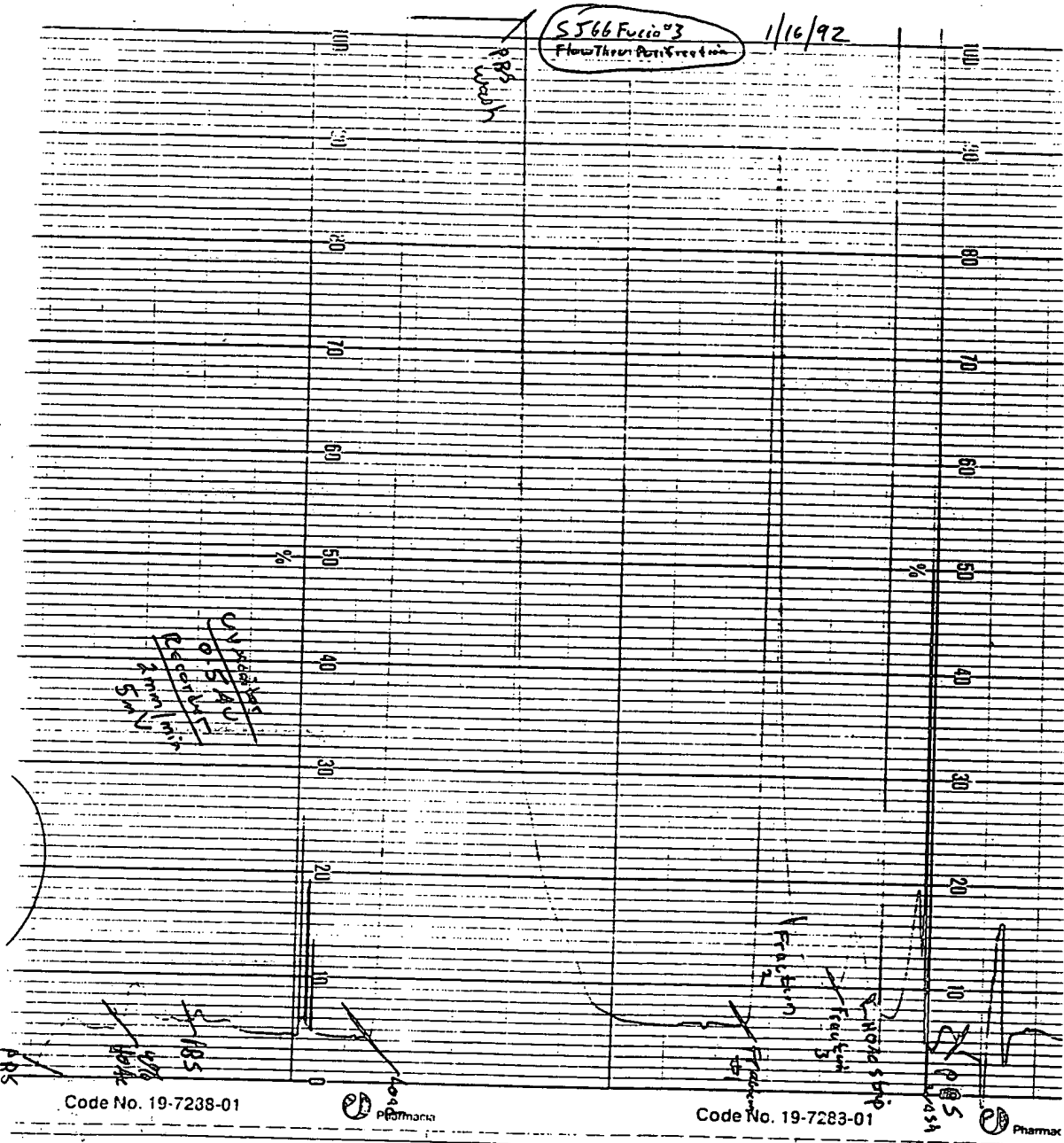
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TITLE 1308F Purification SS66 Fucic #3



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S. S. P. P.

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TITLE 1308F Purification SJ66 Fucia #3 and #1

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Assay:

$$\textcircled{1} \text{ Column Sample: } 4.01 \mu\text{g/ml} \times 640 \text{ ml} = 2.57 \text{ mg}$$

$$\textcircled{2} \text{ Flow Thru: } 0 \mu\text{g/ml}$$

$$\textcircled{3} \text{ Fraction 1: } 101.8 \mu\text{g/ml} \times 11.4 \text{ ml} = 1.16 \text{ mg}$$

$$A_{260} = .002 = 1.44 \mu\text{g/ml}$$

$$\textcircled{4} \text{ Fraction 2: } 10.8 \mu\text{g/ml} \times 8.3 \text{ ml} = 0.09 \text{ mg}$$

$$A_{260} = .026 = 18.6 \mu\text{g/ml}$$

$$\textcircled{5} \text{ Fraction 3: } 6.0 \mu\text{g/ml} \times 11.7 \text{ ml} = 0.07 \text{ mg}$$

- dialyzed  
samples

-- Slower elution rate might be giving a tighter elution, but since this wasn't as heavily loaded as the 1/16 column it is not a fair comparison.

-- Only 51% recovery (1.32 mg) of the input sample was attained

-- Toss fraction #3, since its elution was aided by the addition of 4%

SJ66 Fucia #1 Purification

Will purify the 1308F from the SJ66 Fucia #1 conditioned medium (assayed at  $11.4 \mu\text{g/ml} \times 600 \text{ ml} = 6.8 \text{ mg}$ ). Will elute the column with 3M KSCN to see (1) if it gives a better elution profile and (2) if the product is substantially different than the 5M  $\text{NH}_4\text{OAc}$  PBS eluted material.

-- Rinse G4H IgG-farose column (8ml bed volume) with 60ml PBS, wash with 4% H<sub>2</sub>O<sub>2</sub>, rinse with ~30ml PBS -- pH 7.4 at end.

-- Load Column at ~2.5ml/min (blue-yellow tubing, 500).

-- Wash column with ~100ml PBS, UV trace to baseline.

-- Elute slowly (setting 320) with 3M KSCN. Collect 3 fractions

-- Dialyze fractions 4hrs against 2L PBS.

-- Change buffer, dialyze o/n.

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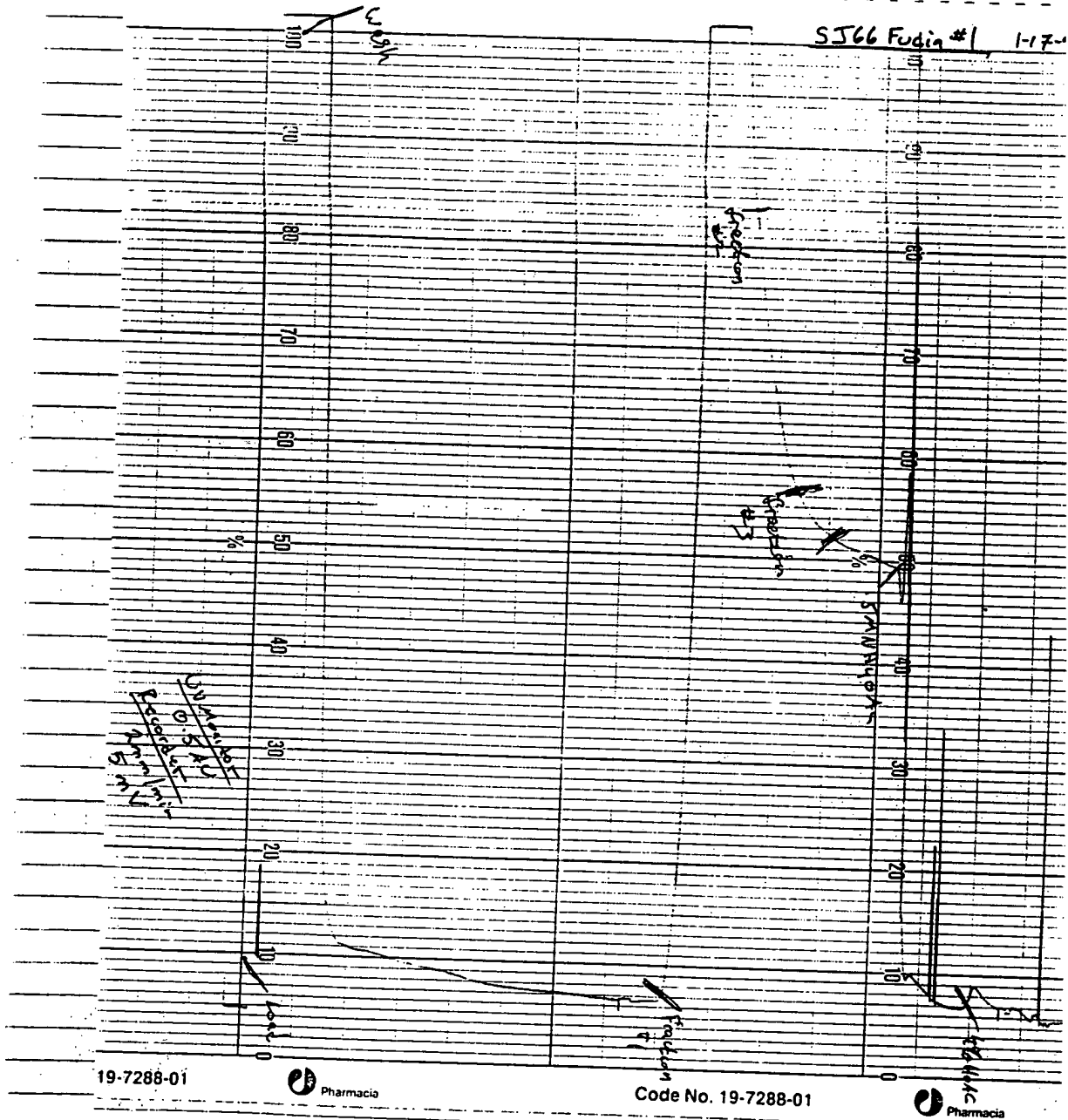
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TITLE 1308F Purification: SJ66 Fucia #1

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D. B. P. P.

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1/1/21

T Page N

TITLE 1308F Purification: SJ66 Fucia #1 and #3

Project No. 98

Book No. 95

From Page No. —

20 January 1992

Assay

① Sample:  $8.92 \mu\text{g/ml} \times 600 \text{ml} = 5.35 \text{mg}$

② Flow Thru #1:  $2.15 \mu\text{g/ml} \times 410 \text{ml} = 0.88 \text{mg}$

③ Flow Thru #2:  $15.23 \mu\text{g/ml} \times 190 \text{ml} = 2.89 \text{mg}$

④ Fraction #1:  $7.47 \mu\text{g/ml} \times 9.5 \text{ml} = 7.1 \text{mg}$

⑤ Fraction #2:  $119 \mu\text{g/ml} \times 9.0 \text{ml} = 1.07 \text{mg}$  — Dialyzed Samples

⑥ Fraction #3:  $26.75 \mu\text{g/ml} \times 6.7 \text{ml} = 0.18 \text{mg}$

-- Even at a slow flow rate the capacity of the column is exceeded, so it is apparent capacity of the column that is a problem and not the flow rate of loading.

-- 8.35 mg were eluted from the column and 3.77 mg flowed through the col. but there were only 5.35 mg in the starting sample. Apparently some of the material "lost" from the SJ66 Fucia #3 runs was never eluted from the col. with 5M NH<sub>4</sub>OH pH 3 or 4 or acetic acid, but it did elute with 3M KOAc. If is the case, then these samples may not be useful since some of the protein them have been subjected to several rounds of acid washing.

21 January 1992

Concentrate samples: SJ66 Fucia #3 fraction #1 (LNB 95-136)

Test Centrprep concentrators (Amicon #4306)

① Make Human IgG Soln:  $0.6 \text{ml} \times 17.8 \text{mg/ml} \rightarrow 42.5 \text{ml} = 25 \mu\text{g/ml}$   
 $\text{OD}_{280} = 0.321 = 229 \mu\text{g/ml}$

② load 11ml  $229 \mu\text{g/ml}$  into centrprep 30 (2.519 mg)

③ Spin 10 min, 2400 RPM, Beckman GPR tabletop centrifuge

④ decant filtrate, spin on additional 9 min as above.

⑤ Remove retentate:  $2.2 \text{ml}$ ,  $\text{OD}_{280} = 1.411$   
 $= 1.01 \text{mg/ml} \times 2.2 \text{ml} = 2.22 \text{mg} = 88\% \text{ recovery}$

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To Page 1

Project No. 78  
Book No. 75

TITLE SJ1308F Purification: SJ66 Fucia #3

Page No.     

Wash Centriscop 30 = PBS:

- (1) load 5ml PBS
- (2) Spin 10', 2400 RPM, decant filtrate + retentate.

SJ66 Fucia #3 G<sub>0</sub>H IgG Agarose Fraction #1 (LWB95-136) Concentration:

- (1) load 7.5ml onto prewashed Centriscop 30, Spin 10', 2400 RPM
- (2) decant filtrate (4.5ml), repeat spin, 4'.
- (3) Remove Retentate: Final volume = 1ml  $A_{280}(1:40) = .076$   
 $= 2.17 \text{ mg/ml} \times 1 \text{ ml} = 91\% \text{ recovery}$

$A_{280} \text{ Retentate (undiluted)} = .006$

SJ66 Fucia #3 G<sub>0</sub>H IgG Agarose Fraction #2 and #3 Concentration (LWB95-136)

- (1) load fraction #2 (8.2ml  $\times$  81mg/ml) + fraction #3 (9.9ml  $\times$  27.8mg/ml) into same centriscop 30 that fraction #1 was concentrated on.
- (2) Spin 10', 2400 RPM, decant 6.5ml filtrate.
- (3) Spin 10', 2400 RPM, decant filtrate (5.5ml) } 14.5ml filtrate total
- (4) Spin 5', 2400 RPM, decant 2.5ml filtrate }  $A_{280} \text{ filtrate} = .003$ , total
- (5) Add SJ66 Fucia #3 FlowThru Purification fraction #1 (LWB95-139) to the retentate cup (11.2ml  $\times$  144mg/ml). Spin 10', 2400 RPM, decant.
- (6) Spin 10', 2400 RPM, decant.
- (7) Spin 5', 2400 RPM, decant.  $OD_{280} \text{ filtrate} = .004$   
Retentate = 1.8ml  $OD_{280} = 1.85 = 1.32 \text{ mg/ml}$   
 $= 2.38 \text{ mg total} = 94\% \text{ recovery.}$

→ Pool SJ66 Fucia #3 fraction #1 concentrate with SJ66 Fucia #3 fraction #2+3/SJ66 Fucia #3 FlowThru fraction #1 concentrate:

$$(2.17 \text{ mg/ml} \times 1 \text{ ml}) + (1.32 \text{ mg/ml} \times 1.8 \text{ ml}) = 3.49 \text{ mg} / 2.8 \text{ ml} = 1.25 \text{ mg/ml}$$

-- .22 micron filter (Amicon #4455) Ab prep, aliquot in 5 - 0.5ml aliquots and one 0.3ml aliquot.

$$-- OD_{280} = 2.12 = 1.51 \text{ mg/ml}$$

Labeled: h1308F  
95-142

Top: 

1-21-92

Tube 1: Jim Tomura 1-22-92  
Tube 2: Charles Riggan 1-23-92  
Tube 3:  
Tube 4:  
Tube 5:  
Tube 6: DSPase To Page N

Invested & Understood by me,

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TITLE 1308F Purification SJ66 Fucia #2

Project No. 98

Book No. 95

From Page No. —

### SJ66 Fucia #2 Purification

I will purify the 1308F from 1/2 of the SJ66 Fucia #2 1/4-1/4 conditioned medium, a higher capacity column and KSCN elution.

- Increase bed volume of the G&H IgG-agarose column to 10ml with fr resin (~~BD~~<sup>20P</sup>) (Sigma 13543 lot 059F-4883). Rinse column with PBS.
- Strip column with 20ml ~~X~~<sup>BD</sup> 3M KSCN, rinse with 50ml PBS.
- Load column, RT, 4ml/min (blue-yellow tube, 790); collect flow thru in 2 aliquots, #1 ~ 280ml and #2 ~ 250ml. Collect the very last 2ml thru in separate tube labeled "Terminal Flow Thru".
- Wash column ~ 70ml PBS to stable baseline; Note: the CV baseline 3M KSCN is ~ 4.5 X that of PBS (PBS baseline @ 6%, KSCN @ 50%). Baseline was adjusted for KSCN, recorder set to 2mV for PBS to be even.
- Elute ~ 3M KSCN, collect 4 fractions of 3-6ml.
- Dialyze fractions against 2L PBS, 4°C, O/N.

22 January 1992

Change dialysis buffer, continue dialysis for 4 hrs.

Remove samples to 15ml polypropylene tubes; read OD of samples and of dialy buffer, blanked against PBS:

① Dialysis buffer - 0.005

Fraction 1: $OD_{280} = .122 = 87 \mu\text{g/ml} \times 2\text{ml} = 174 \mu\text{g}$	Total = 4.96mg
Fraction 2: $OD_{280} = .922 = 659 \mu\text{g/ml} \times 5.2\text{ml} = 3.42 \text{mg}$	
Fraction 3: $OD_{280} = .313 = 224 \mu\text{g/ml} \times 4.0\text{ml} = 894 \mu\text{g}$	
Fraction 4: $OD_{280} = .121 = 86 \mu\text{g/ml} \times 5.4\text{ml} = 468 \mu\text{g}$	

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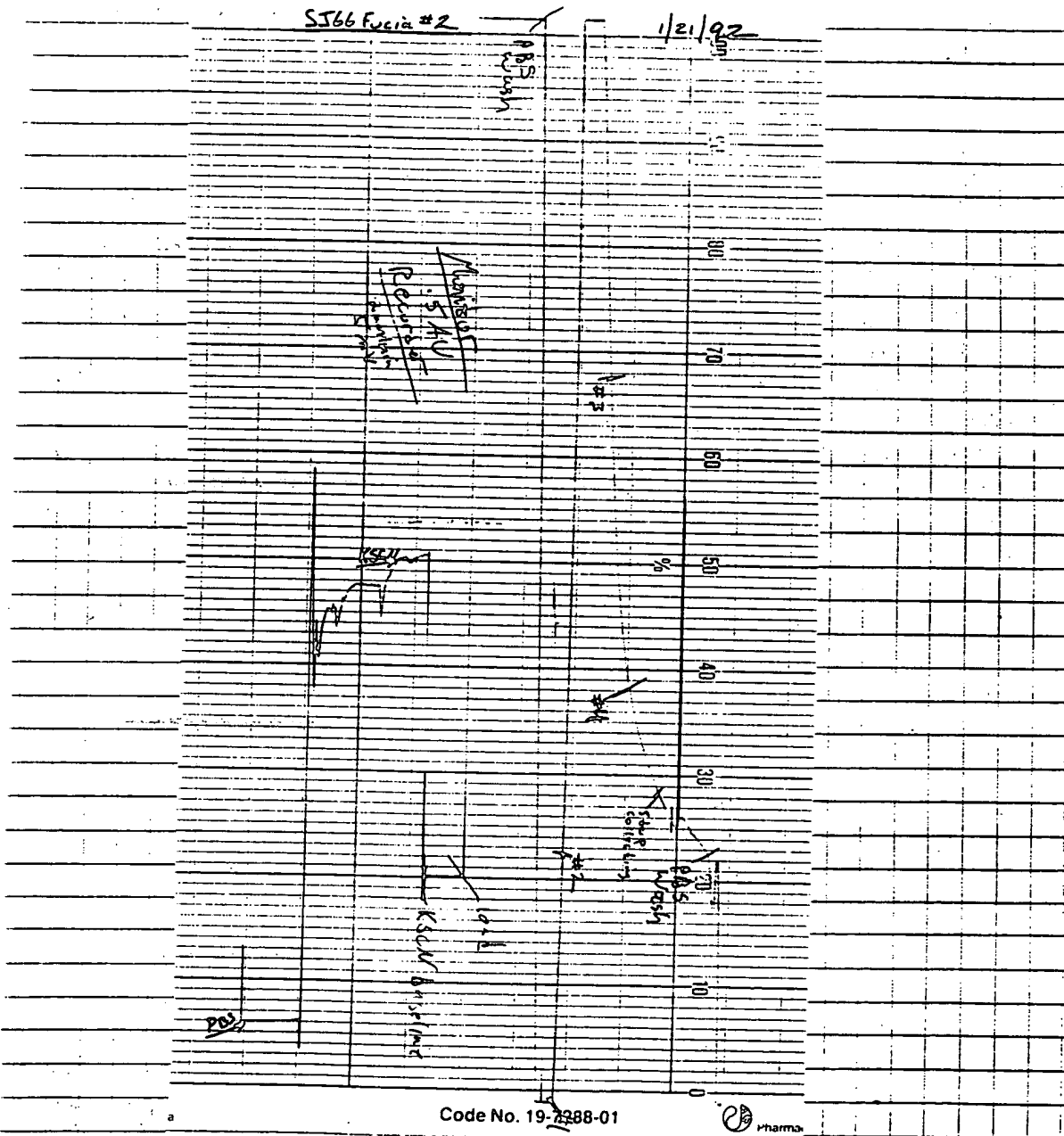
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Book No. 5

TITLE 1308F Purification: SJ66 Fucose #2

Page No. \_\_\_\_\_



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**To Page N**

1/22/95



11  
TITLE 1308F Purification ... J66Fucia #2

Project No. 98

Book No. 95

From Page No. \_\_\_\_\_

Pool SJ66Fucia #2 G94 Ig & agarose fractions #1, 2, 3, 4: 4.96mg/16.6ml

① Spin 10', 2400 RPM, 4°C, Beckman GPCR in Centrifuge 30

② decant 3.4ml filtrate: OD<sub>280</sub> = .004, loss.

③ Spin 10', 2400, 4°C. Decant 3ml filtrate: OD<sub>280</sub> = .002, loss.

④ Spin 15', 2400, 4°C. Decant 3.1ml filtrate: OD<sub>280</sub> = .000, loss.

⑤ Spin 15', 2400, 4°C. Decant 2.7ml filtrate: OD<sub>280</sub> = .000, loss.

⑥ Spin 5', 2400, 4°C. Remove 3.7ml retentate, OD<sub>280</sub> = 1.909 = 1.36mg/ml

$$1.36 \text{ mg/ml} \times 3.7 \text{ ml} = 5.03 \text{ mg}$$

filter sterilize (Gelman #4454, .2µm), aliquot in 6 - 0.5ml and  
1 - 0.3ml aliquots.

$$\text{Read OD}_{280} = \cancel{3.6}^{2.8} \times 1.80 = 1.29 \text{ mg/ml}$$

Label: h1308F  
95-145  
1-22-92

Top: 

Tube 1: Jim Tamara 1-22-  
Tube 2: Charles Riggan  
Tube 3: Gertha Bensen  
Tube 4: Gertha Bensen  
Tube 5: Gertha Bensen  
Tube 6:  
Tube 7: D&P use

23 January 1992

Homogenized 1308F Purification Preparations 1/92

Prep: h1308F  
95-145

Refract: 1.51mg/ml  
Growth Medium 1% Agarose  
2.5% MOPS, pH 7.0 buffer  
2.5% Dith. Monothiuron  
dialyzed against PBS  
Centrifuge 30 concentrated  
Aman filtered

Concentration: 1.51mg/ml

Aliquots: 5 x 0.5ml, 1 x 0.3ml

Distribution: Tube 1 to J.T. 1-22-92  
Tube 2 to CHR 1-23-92

Prep: h1308F  
95-145

Refract: 1.29mg/ml  
Growth Medium 1% Agarose  
2.5% MOPS, pH 7.0 buffer  
2.5% Dith. Monothiuron  
dialyzed against PBS  
Centrifuge 30 concentrated  
Aman filtered

Concentration: 1.29mg/ml

Aliquots: 5 x 0.5ml, 1 x 0.3ml

Distribution: Tube 1 to J.T. 1-22-92  
Tube 2 to CHR 1-23-92

Summary of purification  
for Syn.

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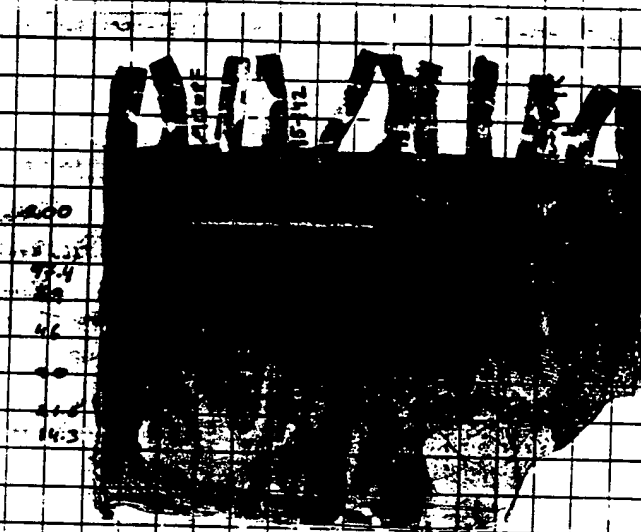
1/22/92

Project No. 98Book No. 75TITLE 1308F Purification Lanes: SJ66 Fused #2, #3Form Page No.     24 January 1992

Load 1 µg each of 95-142 and 95-145 on 4-15% Gradient SDS-PAGE, along w/ 1 µg Mouse 1308F and 1 µg H<sub>2</sub> IgG, k standard. Run 1 hr, 115 volts. Stain 1 hr, destain O/N.

25 January 1992

Change destain, shake O/N.

27 January 1992

Even though by 1000 counts amounts of all 4 gels were loaded (1 µg), there seems to be less protein in the 95-145 lane. Also, 95-145 has a small (100k) contaminant not seen in other lanes.

Note: MSC did RIP against labeled, RSV infected, HEPE cells on 1/28/92 both 95-142 and 95-145 immunoprecipitate RSV-F protein. 95-142 brought down more RSV-F than did 95-145 (equal amounts of MAb used) which can be explained by the discrepancy in concentrations seen in the above gel.

28 January 1992

Note: JT performed a competition ELISA, F protein down, using M1308F and 95-142 and 95-145, detecting with α-Mouse-HRP. Both 95-142 and 95-145 compete with M1308F for binding to RSV-F but with an apparent K<sub>d</sub> ~200 fold lower than M1308F.

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TITLE H308F Purification: SJ66 Fucia #2 + #3

Pr at No. 98  
Book No. 95

From Page No.     

Assay Results (Assay done 1.23.92)

SJ66 Fucia #2 Purification

① Sample: 11.28 mg/ml

② Flow Thru #1: -0-

③ Flow Thru #2: 1.87 mg/ml

Toss These.

④ Final 3ml of Flow Thru: 2.86 mg/ml

⑤ Purified, concentrated H1308F 95-145: 1.01 mg/ml

-- Apparently the capacity of the column is low, since all of the Ab is absorbed out of the first 280ml of sample, but as the loading continued Ab was absorbed by the column.

-- The ELISA shows only 1.01 mg/ml H1308F in the final, concentrated sample whereas by Asso there is 1.29 mg/ml. Part of this discrepancy may be due to the 410kd contaminant.

-- H1308F 95-142 is 1.89 mg/ml by ELISA but only 1.51 mg/ml by Asso. ELISA and Asso readings need to be repeated for both preps.

14 February 1992

Confirmatory ELISA

H1308F 95-142: 3.1 mg/ml (@ 1:40,000 dilution)

H1308F 95-145: 1.60 mg/ml (@ 1:20,000 dilution) } 1.72 mg/ml  
1.84 mg/ml (@ 1:40,000 dilution)

-- These results are quite different than earlier ELISA results and Asso results. Will have to repeat these on another ELISA.

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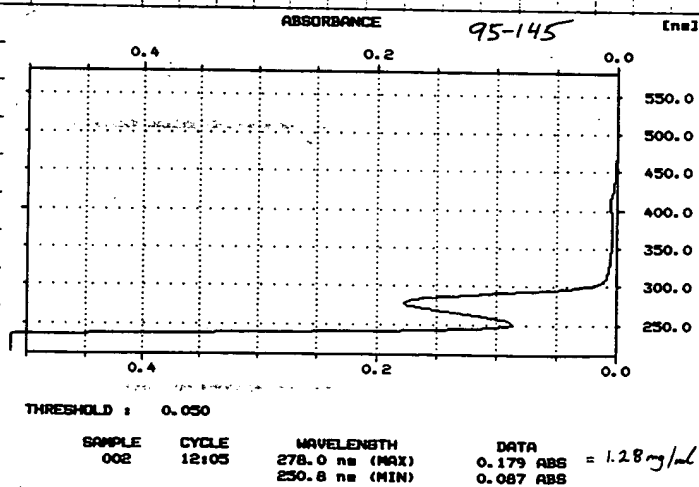
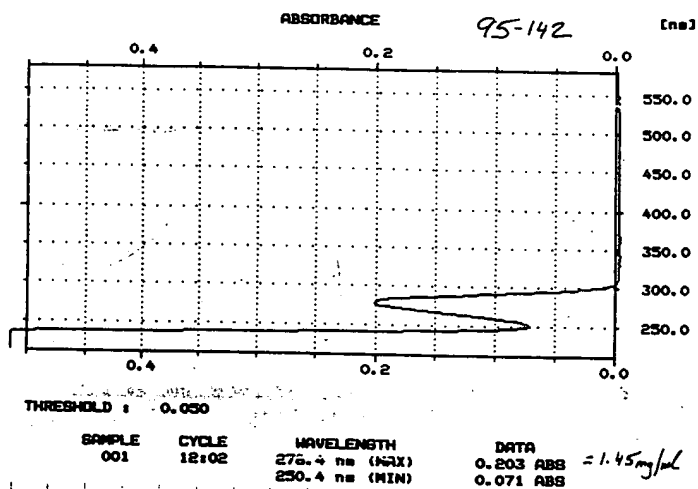
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Project No. 98Book No. 15TITLE H1308F Purification: 95-142, 95-145m Page No.     20 February 1992

Scan 1:10 dilutions of 95-142 and 95-145, 210nm → 880nm:



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## Humanized 1308F Purification Preparations 1/92

ep: h1308F  
95-142

ified: 1/5/92 - 1/21/92 Goat & Human IgG-Agarose  
0.5M  $\text{NH}_4\text{OAc}$  pH 3.0 Eluted  
2M Tris Neutralized  
dialyzed against PBS  
Centriprep 30 concentrated  
2um Filtered

concentration: 1.51 mg/ml

vials: 5 x 0.5 ml, 1 x 0.3 ml

distribution: Tube 1 to JT 1-22-92  
Tube 2 to CHR 1-23-92

ep: h1308F  
95-145

ified: 1/21/92 - 1/22/92 Goat & Human IgG-Agarose  
3M KSCN Eluted  
dialyzed against PBS  
Centriprep 30 Concentrated  
2um Filtered

concentration: 1.29 mg/ml

vials: 6 x 0.5 ml, 1 x 0.3 ml

distribution: Tube 1 to JT 1-22-92  
Tube 2 to CHR 1-23-92  
Tube 3+4 to GB 2-20-92

111

TITLE H1308F Purification 5-142, 95-145

ject No. 98

Book No. 95

From Page No. —

ditate 95-142 & 95-145 Add 4 µg of each 1:10 dilution to 9 tubes (31.25A 95-27.6A 95-142), bring to 40 µl of PBS, add 70 µl 4X Protein sample buffer to each.

ELISA on 95-142 and 95-145:

95-142 : 2.70 mg/ml  $A_{280}$  (22000)  
 95-145 : 1.27 mg/ml 1.45 mg/ml  
 1.28 mg/ml

### Quantitation Results

95-142		95-145	
ELISA	$A_{280}$	ELISA	$A_{280}$
1.89 mg/ml	1.51 mg/ml	1.01 mg/ml	1.29 mg/ml
3.10 mg/ml	—	1.72 mg/ml	—
2.70 mg/ml	1.45 mg/ml	1.27 mg/ml	1.28 mg/ml
$\bar{x} = 2.56$ mg/ml	$\bar{x} = 1.48$ mg/ml	$\bar{x} = 1.33$ mg/ml	$\bar{x} = 1.29$ mg/ml

H1308F95-1  
 = 1.3 mg

The ELISA and  $A_{280}$  for 95-142 are quite different, whereas they agree fairly well for 95-145.

21 February 1992

Went to look at 95-142 and 95-145 on protein gel to see if they are pure and intact still. Run 15% SDS-PAGE:

Lane Sample  
 1 Rainbow Markers  
 2 1 µg H1gG1,k  
 3 1 µg 95-142  
 4 1 µg 95-145  
 5 empty  
 6 3 µg 95-142  
 7 3 µg 95-145  
 8 —  
 9 —  
 10 —



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To Page

Project No. 98

Book No. 1

TITLE CH1129 and H1308F-S71 Purification

Page No.     

Purification  
CH1129 and H1308F-S71

March 1992

Thaw COS supernatants (LNB95-53→56) at 40°C O/N

H1308F-S71

2/11-2/14	37ml @ 2.78 µg/ml = 103 µg	} 326 µg total / 115 ml
2/14-2/17	36ml @ 3.90 µg/ml = 140 µg	
2/17-2/20	42ml @ 1.97 µg/ml = 83 µg	

CH1129

2/11-2/14	26ml @ 4.45 µg/ml = 116 µg	} 375 µg total / 86 ml
2/14-2/17	28ml @ 5.90 µg/ml = 165 µg	
2/17-2/20	32ml @ 2.93 µg/ml = 94 µg	

March 1992

- Pour 2 1ml bed volume GxH IgG-sepharose (Sigma) columns, wash with PBS, load samples by gravity flow (~3ml/min).

- Wash columns w/ PBS

- Elute w/ 3M KSCN (3M in H<sub>2</sub>O, pH ~7.5 unadjusted).

- Collect 3 fractions of H1308F-S71, 4 fractions of CH1129.

- Dialyze fractions 1.5 hr against PBS in microdialyzer.

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D. S. P. P. P.

Date

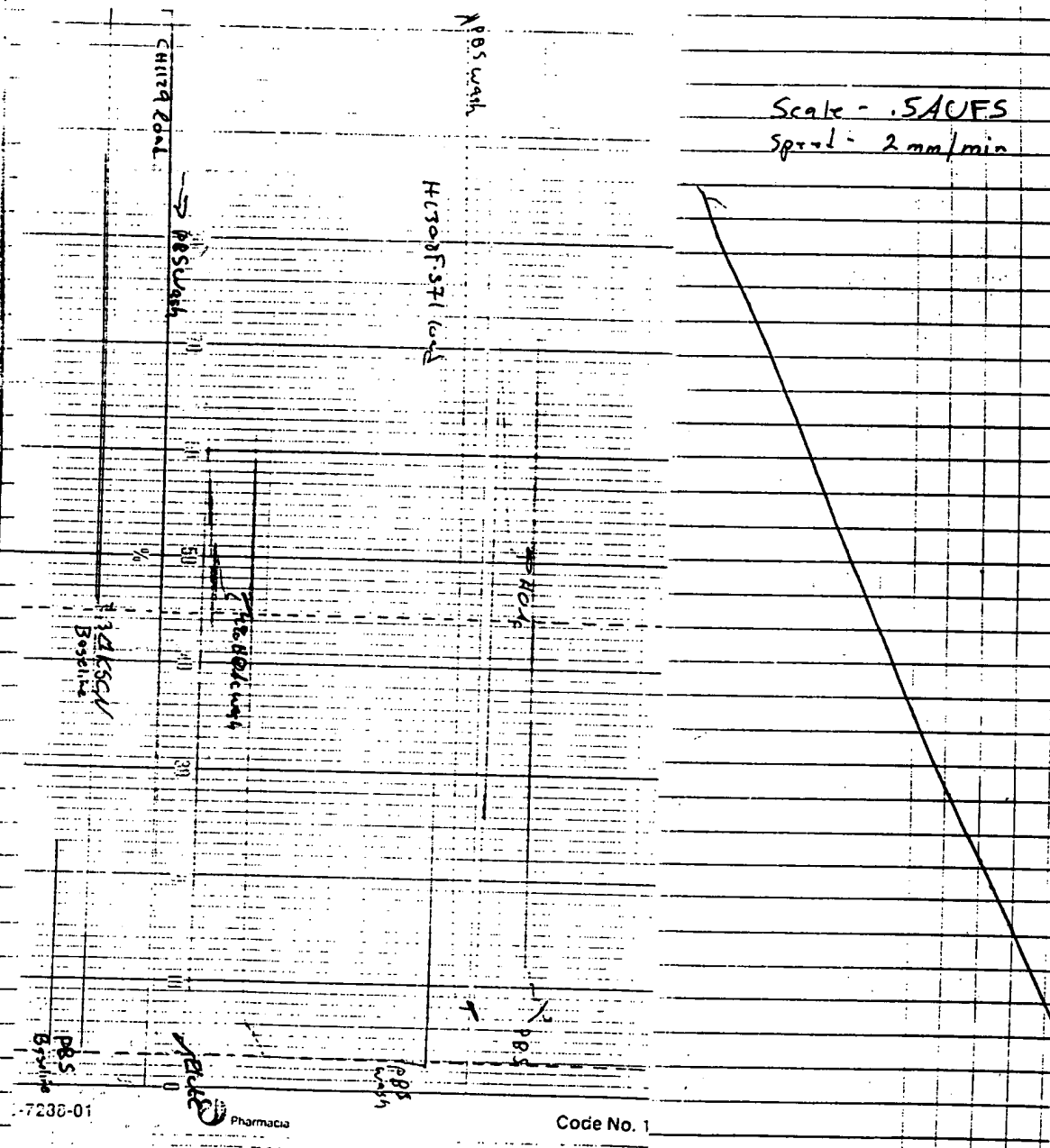
3/2/92

T Page No.

TITLE CH1129 and H1308, 571 Purification

Project No. 98  
Book No. 95

From Page No. \_\_\_\_\_



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Date

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Date

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Project No. 98  
Book No. 5

TITLE CH1129 and H1308F-571 Purification

Page No. 1

March 1992

ELISA

	<u>CH1129</u>	<u>H1308F-571</u>
Column Sample	$4.8 \mu\text{g/ml} \times 86 \text{ ml} = 413 \mu\text{g} (100\%)$	$3.3 \mu\text{g/ml} \times 115 \text{ ml} = 380 \mu\text{g} (100\%)$
Flow Thru	$0.9 \mu\text{g/ml} \times 86 \text{ ml} = 78 \mu\text{g} (19\%)$	$0.66 \mu\text{g/ml} \times 115 \text{ ml} = 80 \mu\text{g} (21\%)$
Fraction 1	$29.2 \mu\text{g/ml} \times 7 \text{ ml} = 20 \mu\text{g} (5\%)$	$0.0 \mu\text{g/ml} \times 7 \text{ ml} = 0 \mu\text{g} (0\%)$
Fraction 2	$24.1 \mu\text{g/ml} \times 9 \text{ ml} = 22 \mu\text{g} (5\%)$	$22.4 \mu\text{g/ml} \times 1.9 \text{ ml} = 42 \mu\text{g} (11\%)$
Fraction 3	$17.8 \mu\text{g/ml} \times 1.5 \text{ ml} = 27 \mu\text{g} (7\%)$	$13.7 \mu\text{g/ml} \times 1.5 \text{ ml} = 21 \mu\text{g} (6\%)$
Fraction 4	$9.4 \mu\text{g/ml} \times 2 \text{ ml} = 19 \mu\text{g} (5\%)$	N/A

CH1129: pool fractions 1, 2, 3, 4; dialyze against 2L PBS 3hr, change buffer, dialyze OK

H1308F-571: pool fractions 2 + 3; dialyze against 2L PBS 5hr, change buffer, dialyze OK

March 1992

Remove samples to tubes; to  $4^\circ\text{C}$ .

March 1992

ELISA (1:500, 1:100)

CH1129:  $9.2 \mu\text{g/ml} \times 6 \text{ ml} = 55.2 \mu\text{g}$

H1308F-571:  $13.8 \mu\text{g/ml} \times 3.5 \text{ ml} = 48.3 \mu\text{g}$

April 1992

Confirmatory ELISA: 2nd run Filter CH1129; 9.77  $\mu\text{g/ml}$

Project No. 57.98

Book No. 61

TITLE MAb Western: 69, 47, 98-6, CH129, H1129, H1308F-SZ

Page No. \_\_\_\_\_

MAb Western

April 1992

Transfer 0.5 mg each MAb to a clean microfuge tube:

694 (pST106/107 #2, 4.6.92)

98-6 (MEU98-6H #2, 4.6.92)

447 (44713 4.6.92)

H1129 (5X transfection, 5.34.92, LNB95-69) 2 mg/ml

CH1129 (pS186, 3.12.92, LNB95-67) 2.37 mg/ml

CH1129, purified (LNB95-150) 9.2 mg/ml -- 54  $\mu$ l + 200  $\mu$ l PBS

H1308F-SZ1, purified (LNB95-150) 13.8 mg/ml -- 36  $\mu$ l + 200  $\mu$ l PBS

H1308F-6K-F1 (2.5X + ex pooled transfections, LNB118-70, 3.20.92)

H1308F, purified (LNB95-145, from 1:10 dilution 2.20.92) 13 mg/ml -- 4  $\mu$ l + 200  $\mu$ l PBS

COS Mock sample - 1 ml

April 1992

Add 50  $\mu$ l Panzerbin cells to each tube, rotate 4°C 1.5 hr.

Spin cells, decant, resuspend in 0.5 ml 1M buffer

Spin, decant, resuspend in 200  $\mu$ l RIPA

Spin, decant, resuspend in 200  $\mu$ l RIPA, transfer to new tube

Spin, decant.

Resuspend in 100  $\mu$ l 1X sample loading buffer

Boil 10'

Spin 5'

Load 25  $\mu$ l into Daiichi 4-20% gradient SDS-PAGE:

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TITLE MAB Western

Project No. 83.98

Book No. 95

From Page No.     

Lane	Sample
1	low stds
2	COS Mock
3	447
4	694
5	98-6
6	H1129
7	CH1129
8	CH1129, purified
9	H1308F-66-781
10	H1308F-571 purified
11	H1308F purified, 95-145
12	empty



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TITLE 1308F Conditioned Medium: COSP roject No. 98  
Book No. 95From Page No.     1308F/COS Transfection 8/9/91-- Conditioned Medium12 September 1991Transfection Date 8/9/91Plasmid SJ60/SJ61

serum	Harvest Date	Time on cells	Volume	Antibody Concentration	Total Antibody
FBS	8/12/91	72 Hr	75ml	492 ng/ml	36.7 $\mu$ g
SF	8/14/91	48 Hr	15ml	64 ng/ml	1 $\mu$ g
NS	8/15/91	48 Hr	30ml	283 ng/ml	8.5 $\mu$ g
FBS	8/15/91	48 Hr	30ml	322 ng/ml	9.7 $\mu$ g
NS	8/19/91	72 Hr	120ml	238 ng/ml	28.6 $\mu$ g
NS	8/21/91	72 Hr	120ml	155 ng/ml	18.6 $\mu$ g

-- Total Antibody = 103.1  $\mu$ gThaw seps, combine FBS containing and combine NS containing seps:

"Stored" volumes by C. Schmidt:

FBS	46.4 $\mu$ g / 105 ml = .44 $\mu$ g/ml
NS	55.7 $\mu$ g / 270 ml = .206 $\mu$ g/ml
Mock (FBS)	77 ml

Combine Seps, measuring  $\pm$  25ml pipet:FBS

8/15/91 - 27ml	} remove .5ml aliquot
8/12/91 - 26ml + 39ml	
92ml	

NS

8/15/91 - 26ml	} remove .5ml aliquot
8/19/91 - 112ml	
8/21/91 - 98ml	
236ml	

TO 4°C O/N

To Page

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Project No. AB  
Book No. 12

TITLE 1308F Conditioned Medium iCOS

m Page No.     

13 September 1991

Filter 3.5 ml of each (Mock, NS, FBS) supe through an  
Acrodisc 0.2  $\mu$ m Low-Protein-Binding syringe filter to  
sterilize (product # 4192, Gelman Sciences).

-- Remove 0.5 ml, give remaining 3 ml to C. Riggins  
for virus neutralization assay.

ELISA on pool'd supe's, pre- and post-filtering

-- ABTS development very slow; read after ~ 12 min of developing  
-- no development in IgG standard lanes, very little in IgG  
lanes. Develop another 10' -- still poor development

-- Wash plate 1X  $\pm$  PBS-Tween, develop with TM Blue; stop  
with 4.5 M H<sub>2</sub>SO<sub>4</sub>. Much higher development of the  
IgG lanes, still very poor development of the IgG<sub>1</sub> lanes

Generate standard curve using TM Blue data, standards 0  $\rightarrow$  6.75  
correlation coefficient = .998  
Slope = 0.80

Mock = 0

Mock filtered = 0

1308F NS = 16.2 ng/ml

1308F NS filtered = 16.7 ng/ml

1308F FBS = 31.3 ng/ml

1308F FBS filtered = 31.6 ng/ml

These results are way lower  
than expected from previous  
assays (by G. Bensal / To Ruiz).

An error must have been made  
in diluting the standards --  
Hrry it's Friday the 13<sup>th</sup>  
so what do you expect?!!

nessed & Understood by me,

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Date

11/11/91

To Page N

TITLE 1308F Conditioned Medium: (CS) (repeat of 9/13) Project No. .98  
 book No. 95

From Page No. 1308F/CS Transfection (8/9/91)  
Conditioned Medium - Pre and Post Filtering

9/16 September 1991  
 -- repeat of assay, 9/13/91.

Standards

- 3 sets: ① Ig G<sub>1</sub> from T. Ruiz  
 ② Ig G (diluted to 100 ng/ml) on 7/10/91 from G. Bernal  
 ③ Ig G received from Gopal and diluted to 1 ng/ml on 7/12/91.

Results

Standard Curves

<u>Ig G<sub>1</sub></u>	<u>Ig G (from 9/10/91)</u>
0 - .066	0 - .074
1.56 - .075	1.56 - .085
3.125 - .090	3.125 - .110
6.25 - .125	6.25 - .141
12.5 - .187	12.5 - .258
25 - .301	25 - .461
50 - .547	50 - .784
100 - .968	100 - 1.298
slope = .0091	slope = .0125
Correlation Coefficient = .9994	Correlation Coefficient = .9955

-- new Ig G standards are way off scale!! Looking back at the spec.  
 the sample stock solution is actually 17.8 ng/ml!!

<u>#</u>	<u>#</u>	<u>Ig G<sub>1</sub> stds</u>	<u>Ig G stds</u>	<u>% of Ig G<sub>1</sub></u>
1308F FBS Pool		878.4 ng/ml	586.9 ng/ml	50%
1308F FBS Pool - Filtered		871.8 ng/ml	582.1 ng/ml	50%
1308F NS Pool		419.9 ng/ml	267.9 ng/ml	57%
1308F NS Pool - Filtered		370.6 ng/ml	232.1 ng/ml	60%
9/16 SJ66 Supr #3 -		648.8 ng/ml	437.2 ng/ml	48%
9/16 SJ66/SJ61 Supr #8 -		890.1 ng/ml	612.7 ng/ml	45%

Project No. <sup>98</sup>

Book No. 45

TITLE 1308F/COS Quantitation 9/13/91 X1

n Page No. 9/13/91

5566 and 5560/61 Transfection

September 1991

Coat plate: 5ug/well CoH IgG Fr. (DSP stock) in Carbonate buffer  
-40C c/v

September 1991

Dilute Standards: Ig G<sub>1</sub>, Ig G (Immunology), Ig G (DSP stock)

Ig G<sub>1</sub>

Slope = .0131  
C. Coef. = .9972

1:20	$\bar{x}$	1:40
0	0	0
0	0	0
644	636	627
822	841	860
615	605	584
536	595	603
569	550	530
316	846	875
723	718	707
830	813	796
877	846	814
786	861	736

Ig G

(Immunology)

Slope = .0206  
C. Coef. = .9945

1:20	$\bar{x}$	1:40
0	0	0
0	0	0
382	364	345
496	494	492
364	344	323
345	337	329
335	309	282
492	497	502
436	416	395
501	477	452
531	497	463
473	507	541

Ig G (DSP)

Slope = .0195  
C. Coef. = .9943

1:20	$\bar{x}$	1:40	%
0	0	0	
0	0	0	
418	394	370	8
544	539	534	9
397	372	346	8
377	365	352	9
365	333	300	9
540	512	544	9
477	452	426	9
550	519	488	9
583	542	501	9
518	553	588	9

There is an 8-9% difference between Immunology's Ig G standards and my Ig G standards; mine should be fine to use hereafter.

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T Page N

11  
TITLE 1308F/COS 9/13/11 Xfection

Proc t No. .98  
Book No. 95

From Page No.	IgG <sub>1</sub>	IgG	% of
1- Mock	0	0	—
2- Mock	0	0	—
3- SJ66	636	379	68%
4- SJ66	841	517	63%
5- SJ66	605	359	69%
6- SJ66	595	351	70%
7- SJ66	550	321	71%
8- SJ60/61	846	520	63%
9- SJ60/61	718	434	65%
10- SJ60/61	813	498	63%
11- SJ60/61	846	520	63%
12- SJ60/61	861	530	62%

Average 1308F Production		
<del>SJ66</del>	IgG <sub>1</sub>	IgG
SJ66	645 ng/ml	385 ng/ml
SJ60/61	817 ng/ml	500 ng/ml

During the first 72 hrs. of production after the transfection, it appears that the co-transfection (S) yields about 28% more 1308F than the single transfection of SJ66.

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T Page No.



Project No. .98Book No. 75TITLE 1308F/LOS 9/191 x fractionom Page No. 9/13/91 x fraction, 9/16-9/20 Super Quantitation

20 September 1991

Dilute IgG<sub>1</sub> and DSP's IgG standards.

Run assay - standard curve calculation via Softmax software.

COS samples, T150's, seeded 9/16/91; pooled:

SS

Sample	IgG <sub>1</sub>	IgG	%A	NS/FBS	
SJ66-FBS	359 ng/ml	173 ng/ml	75%	IgG <sub>1</sub>	IgG
SJ66-NS	501 ng/ml	237 ng/ml	114%	140%	137%
SJ60/61-FBS	283 ng/ml	141 ng/ml	101%	120%	118%
SJ60/61-NS	340 ng/ml	167 ng/ml	104%		

1308F Production SJ66: SJ60/61		
	IgG <sub>1</sub>	IgG
FBS	127%	123%
NS	147%	142%

\*

Note: P100's were scraped and seeded into T150's c 9/16/91; cells had low viability and were not very dense on

① Cells seem to be producing more 1308F in 10% NS than in 10% F

② Later in the transfection (secreting) the SJ66 gives higher production than the cotransfected SJ60 and SJ61. This could be due to unequal amounts of SJ60 and SJ61 in the cells.

T Page No.

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Date

9/20/91

11  
TITLE 1308F/COS 9/13/91 Transfection

Pro No. 98  
Book No. 95

From Page No.

9/13/91 Cos Transfection: ELISA

9/27 - 9/30 Samples

30 September 1991

Dilute IgG1 and IgG Standards (10 ng/ml ~~with~~ Starts from 9/20/91)

Samples: Mock NS

Mock FBS

SJ66 NS

SJ66 FBS

SJ66/61 NS

SJ66/61 FBS

-- 2 Samples from T. Ruiz, 1:50 and 1:500.

### Results

Standards are 10 fold higher than what is shown on cov. sheet --- mis-diluted first standard 1:10 (1 ng/ml) instead of 1:100 (100 ng/ml).

IgG1 Standards 0-125 ng/ml

Slope = .005

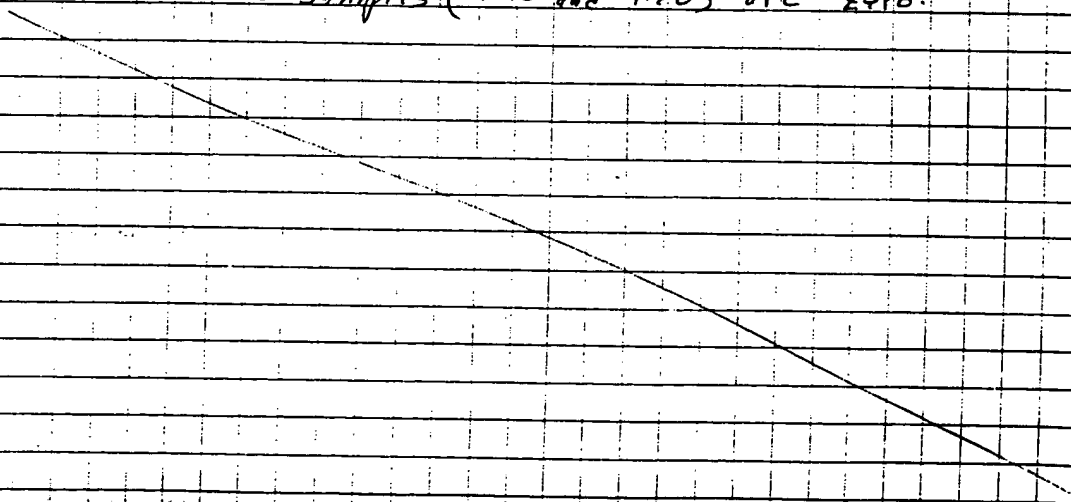
Correlation Coefficient = .997

IgG Standards 0-125 ng/ml

Slope = .007

Correlation Coefficient = .991

-- all Cos samples (1:10 and 1:20) are Zero!



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Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE 1308F Quantita. : Purification #1

Page No. \_\_\_\_\_

1308F 8.9.91 Purification: ELISA

2 October 1991

Dilute Tony Ruiz's IgG1 to 100ng/ml, serially dilute 1:2

Rec'd new Human IgG1k (Sigma # I3889, lot # 8CH8965).

-- Dilute in 1ml PBS (~1mg/ml)

-- dilute an aliquot 1:10 (100 $\mu$ l  $\rightarrow$  900 $\mu$ l PBS), read  
A<sub>280</sub>/A<sub>260</sub>:

$$A_{280} = .189$$

$$A_{260} = .133$$

$$\text{Protein concentration} = 1.5(.189) - .75(.133) = 184 \mu\text{g/ml}$$

-- Dilute to 100ng/ml, serially dilute 1:2

Extinction coefficient for immunoglobulins is 1.4

$$\therefore 1\text{L } 1:10 \text{ dilution} = \frac{.189}{1.4} = .135 \text{ mg/ml} = 135 \mu\text{g/ml}$$

$$\text{Stock IgG} = 1.35 \text{ mg/ml}$$

Aliquot 50 $\mu$ l/Tube @ 1.35 mg/ml IgG1k

-- Store @ -20 $^{\circ}$ C.